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# The Effects of Large Osmolality Variations on the Gastric Mucosal Ion Permeability

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## Abstract

FRENNING B *The effects of large osmolality variations on the gastric mucosal ion permeability* Acta physiol scand 1974 *90* 1—13

NaCl solutions were instilled into ligated unstimulated whole stomach pouches in cats for 30 min On instillation of distilled water or 310 or 620 mM NaCl the net fluxes of NaCl were roughly proportional to the mean concentration difference between the gastric lumen and the blood On instillation of more than 620 mM NaCl the absorption was larger than expected from this proportionality a result believed to be due to an increase in the permeability of the gastric mucosa on instillation of such hyperosmotic solutions Instillation of distilled water or 310 or 620 mM NaCl had only minor effects on the net flux of hydrogen ions out of the stomach during subsequent instillations of 170 mM HCl in comparison with the controls On instillation of HCl subsequent to an instillation of 620 mM NaCl the net fluxes of sodium ions and water into the stomach were significantly increased over the control level and there was a net influx of chloride ions instead of a net efflux. This result was probably due to secretion of a NaCl containing fluid On instillation of 170 mM HCl subsequent to an instillation of 930 or 1550 mM NaCl the net effluxes of hydrogen ions also were significantly increased over the control levels The concentration of NaCl in the instilled solution needed to obtain an effect on the gastric mucosal ion permeability both during the instillation of NaCl and on subsequent instillation of 170 mM HCl was thus larger than 620 mM

It has been claimed previously that hyperosmotic solutions of some nonelectrolytes (glucose sucrose and urea) increase the ion permeability of the gastric mucosa as an effect of their hyperosmolality (Altamirano 1969) In the expts that led to this conclusion the net fluxes of sodium and chloride ions in particular into the stomach on instillations of hyperosmotic solutions were significantly increased over the control levels Concomitant with the increase in net influxes of ions there was a net movement of water into the stomach Similar results have been obtained on intragastric instillation of 170 mM HCl subsequent to an instillation of hyperosmotic L(+) or D(−) lactic acid which had no effect either on the net efflux of hydrogen ions from the stomach or on the mucosal surface morphology (Frenning 1972 a) Even on instillation of distilled water immediately subsequent to hyperosmotic solutions (Altamirano 1969) the occurrence of gastric secretion was in

dicated, the net flux of water out of the stomach being smaller at that time than at a later time point Thus the results of Altamirano (1969) do not necessarily imply that the ion permeability of the gastric mucosa was increased Davenport (1968) has shown however that 1 2 or 4 M solutions of urea increase the gastric mucosal permeability to ions but he claimed that this effect was due to a specific action of urea on the organization of lipoprotein layers and hydrogen bonds As it is generally accepted that urea easily moves into and out of cells it may be claimed that the effect of urea solutions on the gastric mucosa is probably not entirely related to their osmolality

It was considered that further work was required to determine whether hypo or hyperosmotic solutions *per se* have any effect on the gastric mucosal permeability to ions To expose the gastric mucosa to distilled water or to hyperosmotic solutions of NaCl and examine its permeability to ions for any changes was regarded as a suitable approach to this problem

A study was made to find out whether or not the absorption of NaCl from the stomach was proportional to the mean difference in concentration of NaCl between the gastric lumen and the blood (A deviation from a degree of absorption in proportion to the mean concentration difference would presumably indicate an effect on the gastric mucosal permeability to ions)

In an attempt to establish roughly the extent to which net movement of fluid and of electrolytes contributed to the osmolality regulation of the gastric contents the changes in concentration and the net fluxes of sodium and chloride ions on instillation of distilled water or hyperosmotic solutions of NaCl of different concentrations were examined

Finally the effect of an instillation of distilled water or hyperosmotic NaCl on the changes in concentration and net fluxes of electrolytes on subsequent instillations of 170 mM HCl was studied

## Methods

The expts were performed on cats with a mean weight of 2 7 kg (range 1 6—4 4 kg n = 25) which were fasted for at least 18 h previously but had free access to water Anesthesia was induced with Fluothane® (Halothane®) or chloroform and maintained with chloralose 70 mg/kg b wt and urethane (0 2—0 6 g) both given i v The abdomen was opened by a midline incision and the stomach was isolated by ligatures at the cardia adn the pylorus care being taken not to disturb the blood and nerve supplies to the stomach A glass cannula was inserted in the pyloric portion of the stomach and the abdomen was then closed The stomach was rinsed with physiological saline and its secretory state was checked for at least 2 h The mean arterial blood pressure (±S E n = 24) was 143±4 mm Hg at the start of the expts and 130±5 mm Hg at the end The body temperature was kept constant at about 38 C (mean± S E = 37 9±0 2 at the start of the expts and 37 9±0 1 at the end) with a rectal temperature controlled heating device

*General experimental procedure*
The volume of the solutions instilled was always 6 ml Initial samples of the instilled solution were taken within 1 min of the start of the instillation At 15 min samples were taken without complete draining of the stomach At most 0 1 ml was taken for analysis of acidity chloride, sodium and potassium After 30 min the stomach was emptied and the volume measured to the nearest 0 1 ml in a measuring cylinder

TABLE I Changes in concentration of electrolytes and in volume during 30 min instillations of 6 ml of 170 mM HCl distilled water or hyperosmotic solutions of NaCl into innervated unstimulated whole stomach pouches in cats HCl is the control period HCl HCl and HCl are periods 0–30 30–60 and 60–90 min respectively after removal of the distilled water or NaCl instillate Mean values ± SE are given

| Experimental period | n | ΔH mM | ΔCl mM | $\Delta Na^+$ mM | $\Delta K^+$ mM | ΔV ml |
|---|---|---|---|---|---|---|
| HCl | | −63±6 | −21±2 | +44±3 | +2.9±0.3 | +0.7±0.2 |
| $H_2O$ | | +4±2 | −53±6 | +45±4 | +2.7±0.3 | −0.1±0.2 |
| HCl | 5 | −74±6 | −26±4 | +46±3 | +2.7±0.1 | +0.4±0.2 |
| HCl | | −75±8 | −27±4 | +49±3 | +2.9±0.2 | +0.6±0.2 |
| HCl | | −67±3 | −18±1 | +48±3 | +3.1±0.3 | +0.5±0.3 |
| 310 mM NaCl | | −4±1 | −61±4 | −63±3 | +2.5±0.2 | +1.4±0.2 |
| HCl | 5 | −74±3 | −26±4 | +47±5 | +2.3±0.1 | +0.7±0.2 |
| HCl | | −71±3 | −25±1 | +48±3 | +2.7±0.1 | +1.0±0.2 |
| HCl | | −67±3 | −23±2 | +45±3 | +2.3±0.2 | +0.5±0.3 |
| HCl | | −68±2 | −19±1 | +45±2 | +3.2±0.3 | +0.1±0.2 |
| 620 mM NaCl | | −2±4 | −210±30 | −233±14 | +5.0±1.3 | +2.4±0.2 |
| HCl | 5 | −91±4 | −22±1 | +67±4 | +3.3±0.3 | +1.0±0.2 |
| HCl | | −74±4 | −23±2 | +51±3 | +2.6±0.2 | +0.9±0.3 |
| HCl | | −73±7 | −22±3 | +50±3 | +2.5±0.3 | +1.0±0.5 |
| $HCl_1$ | | −59±6 | −18±1 | +37±4 | +2.7±0.2 | +0.2±0.1 |
| 930 mM NaCl | | +1±4 | −456±30 | −430±29 | +4.6±1.5 | +2.9±0.3 |
| HCl | 5 | −94±6 | −26±2 | +77±6 | +3.6±0.3 | +1.2±0.2 |
| HCl | | −73±3 | −19±1 | +59±1 | +2.7±0.3 | +1.1±0.1 |
| HCl | | −66±7 | −18±2 | +50±2 | +2.1±0.2 | +1.2±0.2 |
| HCl | | −50±4 | −15±1 | +37±2 | +2.6±0.3 | +0.5±0.3 |
| 1550 mM NaCl | | −3±2 | −954±32 | −894±88 | +3.5±0.1 | +4.2±0.7 |
| HCl | 5 | −114±3 | −30±2 | +88±2 | +3.0±0.2 | +1.8±0.2 |
| HCl | | −92±3 | −29±2 | +69±2 | +3.3±0.2 | +1.6±0.3 |
| HCl | 4 | −83±5 | −25±3 | +61±7 | +3.1±0.3 | +1.5±0.2 |

All the expts were started with a 30-min instillation of 6 ml of 170 mM HCl *6 ml of* distilled water or of 310 620 930 or 1550 mM NaCl was then instilled for an equal length of time Finally two or three 30 min instillations of 170 mM HCl were given The first instillation of HCl is designated $HCl_1$ (also referred to as the control period) the second as $HCl_2$ and so forth

The *changes in concentration* of electrolytes reported comprise the differences between the concentrations in the final and the initial samples The *changes in volume* of the instilled solutions comprise the differences between the final and instilled volumes and are corrected for sample volume The *net fluxes* of ions reported comprise the differences between recovered and instilled amounts The expression *net efflux* is used to denote a net flux out of the stomach and *net influx* a net flux into the gastric lumen

*Analysis*

*Acidity determination* 0.01 or 0.05 ml samples were diluted with 5 ml distilled water and titrated with 10 mM NaOH (indicator bromthymol blue) The smaller samples were taken when 620 mM or more concentrated NaCl solution was used

*Chloride* was determined electrometrically on the same samples as were used for acidity determination (Autoburette Unit type ABU12 pH meter type PHM 26c Radiometer Copenhagen Denmark) 5 mM $AgNO_3$ was used for titration

*Sodium and potassium* were determined by flame photometry (Flame photometer Eppendorff Netheler and Hintz GmbH Hamburg) after dilution of 0.01 or 0.05 ml samples in deionized water

The coefficient of variation for sodium determination was ± 2 at 100 mM those for the acidity and chloride determinations were smaller (The coefficients were determined from analysis of 10 samples from the same solutions)

TABLE II The mean net fluxes of electrolytes ± SE in the same expts as presented in Table I The mean electrolyte output in the 30 min preceding the start of the experiments (basal output) is also given

| Experimental period | n | $H^+_t$ µeq/30 min | $Cl^-_t$ µeq/30 min | $Na^+_{et}$ µeq/30 min | $K^+_{et}$ µeq/30 min | ΔV ml |
|---|---|---|---|---|---|---|
| Basal output | 5 | 9 ± 2 | 84 ± 7 | 71 ± 4 | 6 ± 1 | 0 5 ± 0 0 |
| HCl | | −403 ± 27 | −4 ± 39 | +363 ± 36 | +31 ± 2 | +0 7 ± 0 2 |
| $H_2O$ | 5 | +62 ± 10 | +370 ± 31 | +286 ± 25 | +20 ± 2 | −0 1 ± 0 2 |
| HCl | | −466 ± 24 | −111 ± 26 | +329 ± 20 | +23 ± 2 | +0 4 ± 0 2 |
| $HCl_3$ | | −428 ± 41 | −34 ± 35 | +388 ± 32 | +24 ± 1 | +0 6 ± 0 2 |
| Basal output | 5 | 5 ± 3 | 62 ± 11 | 48 ± 13 | 4 ± 1 | 0 4 ± 0 1 |
| HCl | | −428 ± 17 | −41 ± 32 | +376 ± 21 | +26 ± 3 | +0 5 ± 0 3 |
| 310 mM NaCl | | | −118 ± 87 | −65 ± 90 | | +1 4 ± 0 2 |
| HCl | 5 | −458 ± 17 | +38 ± 21 | +467 ± 20 | +25 ± 1 | +0 7 ± 0 2 |
| HCl | | −370 ± 18 | +24 ± 25 | +399 ± 26 | +22 ± 2 | +1 0 ± 0 2 |
| $HCl_4$ | | −357 ± 16 | −19 ± 34 | +334 ± 30 | +17 ± 2 | +0 5 ± 0 3 |
| Basal output | 2 | 4 | 80 | 72 | 5 | 0 5 |
| HCl | | −452 ± 59 | −82 ± 47 | +339 ± 17 | +25 ± 3 | +0 1 ± 0 2 |
| 620 mM NaCl | | | −811 ± 122 | −661 ± 132 | | +2 4 ± 0 2 |
| HCl | 5 | −530 ± 39 | +94 ± 49 | +569 ± 72 | +28 ± 4 | +1 0 ± 0 2 |
| HCl | | −422 ± 38 | +35 ± 64 | +432 ± 40 | +23 ± 2 | +0 9 ± 0 3 |
| $HCl_4$ | | −314 ± 45 | +37 ± 41 | +416 ± 34 | +24 ± 3 | +1 0 ± 0 5 |
| Basal output | 5 | 19 ± 7 | 159 ± 30 | 131 ± 29 | 10 ± 2 | 0 9 ± 0 2 |
| HCl | | −451 ± 38 | −103 ± 20 | +307 ± 22 | +26 ± 2 | +0 2 ± 0 1 |
| 930 mM NaCl | | | −1429 ± 56 | −1693 ± 92 | | +2 9 ± 0 3 |
| HCl | 5 | −672 ± 28 | +96 ± 45 | +770 ± 46 | +34 ± 3 | +1 2 ± 0 2 |
| HCl | | −521 ± 19 | +4 ± 19 | +518 ± 9 | +30 ± 3 | +1 1 ± 0 1 |
| HCl | | −433 ± 38 | +36 ± 32 | +497 ± 13 | +25 ± 2 | +1 2 ± 0 2 |
| Basal output | 4 | 15 ± 6 | 84 ± 17 | 66 ± 13 | 3 ± 1 | 0 5 ± 0 1 |
| HCl | | −302 ± 47 | +1 ± 42 | +298 ± 13 | +25 ± 5 | +0 5 ± 0 3 |
| 1550 mM NaCl | | | −4249 ± 486 | −3992 ± 513 | | +4 2 ± 0 7 |
| HCl | 5 | −671 ± 21 | +146 ± 44 | +854 ± 51 | +28 ± 2 | +1 8 ± 0 2 |
| HCl | | −512 ± 29 | +92 ± 32 | +603 ± 23 | +29 ± 2 | +1 6 ± 0 3 |
| $HCl_4$ | 4 | −460 ± 17 | +70 ± 36 | +567 ± 45 | +28 ± 2 | +1 5 ± 0 2 |

## Results

*Net movement of fluid and electrolytes on instillation of distilled water and hyperosmotic solutions of NaCl*

The changes in volume and in the concentration of hydrogen sodium potassium and chloride ions found on instillation of distilled water and of NaCl solution of different concentrations for 30 min into unstimulated whole stomach pouches are presented in Table I The corresponding net ion fluxes are given in Table II

In distilled water instillates( Fig 1 Table I and II) increases in the sodium potassium and chloride ion concentrations were observed The hydrogen ion concentration increased in some expts and decreased in others resulting in a minor mean increase From the mean increments obtained in the preceding and following HCl instillations it could be estimated that about 0 4 ml was added to the instillate by *secretion* This probably explains why the mean decrease in volume obtained on instillation of water was only 0 1 ml

On instillation of 310 mM NaCl (Fig 2 Table I and II) both the sodium and the chloride ion concentration decreased but the net efflux (out of the stomach)

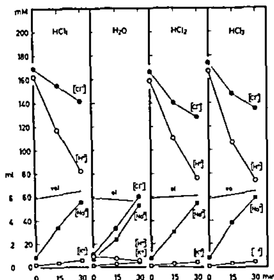



Fig. 1. The result of an expt. in which 170 mM HCl was instilled into a ligated unstimulated cat stomach once before and twice after an instillation of distilled water.

of NaCl was small and comprised only a few per cent of the amount instilled. There was a mean increase in volume by 1.4 ml. Thus the decreases in concentration of sodium and chloride ions were due mostly to dilution.

When 620, 930 or 1550 mM solutions of NaCl were instilled, larger volume increases and more rapid decreases in sodium and chloride ion concentrations were noted (Fig. 3 and 4, Table I). In these experiments considerable net effluxes of sodium and chloride ions took place (Table II). Thus the decreases in ion concentrations observed were due both to dilution and to absorption of NaCl.

The absorption of NaCl was greater on instillation of 930 or 1550 mM NaCl than was expected from the increase in mean concentration difference over the mucosa, see Fig. 5, in which the net flux of sodium ions on instillation of distilled water or

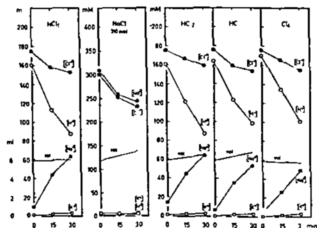



Fig. 2. The result of an expt. in which HCl was instilled once before and repeatedly after an instillation of 310 mM NaCl.

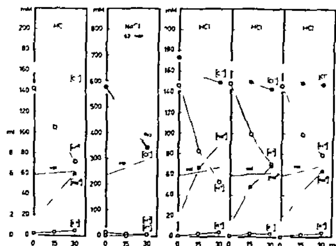


Fig 3 The effect of an instillation of 620 mM NaCl on the changes in concentration of electrolytes on instillation of 170 mM HCl

hyperosmotic NaCl is plotted against the mean sodium ion concentration in the instillate (calculated as the mean of the sodium ion concentrations in the instilled solutions ($Na_0$) and in the final samples ($Na_{30}$)) (Since the sodium ion concentration in the blood can be regarded as constant this plot versus the mean concentration in the instillate also gives information on deviation from proportionality of the net flux of NaCl to the mean concentration difference between the gastric lumen and the blood) The implication of this result is that on instillation of 930 or 1550 mM NaCl the gastric mucosal ion permeability increased (Note that the absorption of NaCl took place against a *flow* of fluid ) The mean NaCl concentration in the instillate needed to clearly demonstrate this effect would be somewhere between 500 and 700 mM (Fig 5) Under the present experimental conditions the mean concentration values of 500 and 700 mM were obtained on instillation of 620 and 930 mM NaCl respectively These values would of course be different with other volume to surface area ratios

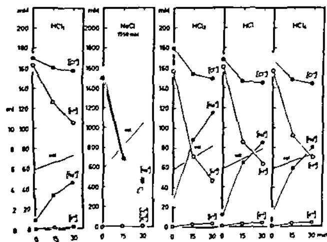


Fig 4 The effect of an instillation of 1550 mM NaCl on the changes in concentration of electrolytes on instillation of 170 mM HCl

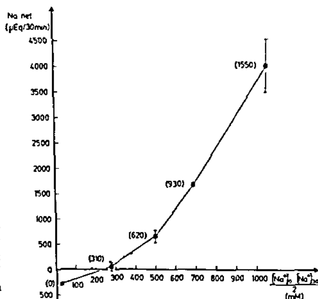



Fig 5 The mean net fluxes of sodium ions ± S E on instillation of distilled water or hyperosmotic NaCl plotted against the mean sodium ion concentration in the instilled solution calculated as the mean of the sodium ion concentrations in the instilled solutions (Na ) and in the final samples (Na30 ) The figures within brackets are the Na0 values (mM) for each point The interrupted proportionality line is constructed from th observations on instillation of distilled water and 310 mM NaCl assuming that there would be no net flux of Na on instillation of 155 mM NaCl and that the output of Na du to secretion was the same in the t o types of experiment

In the control periods only negligible amounts of visible mucus were added to the instillate but on instillation of 620 mM or more concentrated NaCl (as well as on subsequent instillations of HCl) the amount of visible mucus secreted was usually markedly increased and in some expts the increase was very pronounced

In the later instillation periods (*i e* $HCl_3$ and $HCl_4$) minor haemorrhages occasionally occurred

*The effects of an instillation of distilled water and of hyperosmotic solution of NaCl on the mucosal permeability to ions*

On instillation of 6 ml of 170 mM HCl subsequent to a 30 min instillation of *distilled water* (Table II) the net efflux of hydrogen ions (out of the stomach) was slightly but significantly increased ($p < 0.05$) The net efflux of chloride ions also was increased ($p < 0.05$) As the net influx of odium ions (into the stomach) was unchanged the most probable explanation for this result would seem to be that the spontaneous secretion decreased In the following HCl instillation period ($HCl_3$) the net influx of potassium ions decreased significantly below the control level ($p < 0.05$) and the net effluxes of hydrogen and chloride ions returned to the control level

On instillation of 170 mM HCl subsequent to an instillation of *310 mM NaCl* (Table II) the net efflux of hydrogen ions and the net influx of potassium ions were not significantly changed Instead of a small net efflux of chloride ions a small net influx was observed ($p < 0.02$) The net influx of sodium ions was significantly increased ($p < 0.01$) In the two following HCl periods ($HCl_3$ and $HCl_4$) the net effluxes of hydrogen ions decreased lightly below the control level (0 05 < p < 0 1



Fig 6 Surface epithelium from a stomach exposed to 6 ml of 1550 mM NaCl for 30 min and fixed in hyperosmotic fixation solutions *Magnification × 4500* (From Frenning 1973

and $p < 0.02$ respectively) The net influxes of sodium ions regained the control level The net influx of potassium ions was significantly decreased during the last HCl instillation ($p < 0.05$)

After an instillation of *620 mM NaCl* (Table II) the net efflux of hydrogen ions on instillation of HCl was not significantly increased ($0.1 < p < 0.5$) The net influx of sodium ions was significantly increased ($p < 0.05$) A net influx of chloride ions was noted ($p < 0.01$) The net influx of potassium ions was unchanged The mean net efflux of hydrogen ions did not decrease significantly below the control level during the last HCl instillation ($0.1 < p < 0.5$) The net influx of sodium ions was still significantly increased in the last HCl instillation period ($p < 0.05$ In all the periods $HCl_1$—$HCl_4$ the changes in volume were significantly increased over the control level ($p < 0.05$ or less)

In the HCl instillations performed subsequent to an instillation of *930 mM NaCl* ($HCl_1$— $HCl_4$ Table II) the net influxes of sodium ions and the increases in volume were all significantly increased ($p < 0.05$ or less) In $HCl_1$ the net efflux of hydrogen ions was significantly increased ($p < 0.02$) In $HCl_3$ the net efflux of hydrogen ions had regained the control level

The effect of an instillation of *1550 mM NaCl* (Table II) on the transmucosal ion transport on instillation of HCl was essentially the same as that of 930 mM NaCl The net efflux of hydrogen ions was however significantly increased also in $HCl_3$

In the control instillations of 170 mM HCl the net efflux of hydrogen ions usually was larger than the summed net influxes of sodium and potassium ions A net efflux of chloride ions equal to the difference between the net efflux of hydrogen ions and the summed influxes of sodium and potassium ions was obtained When spontaneous

gastric secretion in some of the control periods occasionally caused a decrease in the mean net flux of chloride ions to almost zero the mean net efflux of hydrogen ions as could be expected equalled the summed mean net influxes of sodium and potassium ions (Table II) On instillation of HCl subsequent to an instillation of 620 mM or more concentrated NaCl solution however the summed net influxes of sodium and potassium ions exceeded the net efflux of hydrogen ions (Table II) In these expts there was also a net influx of chloride ions and an increase in volume that was significantly increased over the control level

The results of HCl instillations performed subsequent to an instillation of hyper osmotic NaCl solution in terms of changes in concentration of electrolytes in the instilled solutions are given in Table I see also Fig 2 3 and 4 On instillation of HCl subsequent to an instillation of 620 mM NaCl (*i e* in HCl ) the mean de crease in hydrogen ion concentration was significantly increased from control level ($p < 0.05$) though the net efflux of hydrogen ions was not The same occurred also in the 2nd instillation of HCl performed after an instillation of 930 mM NaCl (*i e* in $HCl_3$) and in the last HCl instillation after exposure of the stomach to 1550 mM NaCl (*i e* in $HCl_4$) In all these periods the mean net influx of sodium ions and the mean increase in volume during the instillations were significantly increased Thus on instillation of HCl subsequent to exposure of the gastric mucosa to hyperosmotic NaCl an increased net movement of sodium chloride and water into the stomach was observed before the net efflux of hydrogen ions increased and this also per sisted when the latter had returned to normal In both cases the hydrogen ion con centration in the instilled HCl was reduced as an effect of dilution

## Discussion

From the work of Hogben (1955) on frog gastric mucosa *in vitro* we know that the gastric mucosa is permeable to sodium ions both in the mucosal (secretory) to serosal (nutrient) direction and the reverse In short circuited mucosa in the absence of a chemical gradient the unidirectional sodium fluxes were equal and thus there was no active transport of sodium Later it has been reported (*e g* by Kitahara 1967) that isolated mammalian gastric mucosae transported sodium actively from the mucosal side to the serosal side As the expts were performed at the same oxygen tension as was used for the frog gastric mucosae *in vitro* and it is known that very high oxygen tensions are needed to minimize lactate production from isolated mam malian stomachs (Davenport and Jensen Chavre 1950) this result may have been due to slight hypoxia of the mucosae This is supported by the finding that slightly hypoxic frog gastric mucosa *in vitro* transport sodium ions actively in the same direc tion (Flemstrom 1971 a) As early as in 1939 Teorell reported a net influx of sodium ions on instillation of HCl into non secreting stomachs On instillation of 7 ml of approximately iso osmotic solution of NaCl and KCl containing $Na^+$ into non secreting cat stomachs (thus with about the same volume to surface area ratio as in the present experiments) the decrease in specific activity was in the order of

20 per cent per h (Nordgren 1963) However some authors claim that the gastric mucosa is very poorly permeable to sodium ions and have introduced the concept of a sodium barrier in this mucosa The reason for this opinion is that the sodium insorption *i e* the unidirectional efflux of sodium ions, expressed as per cent of amount instilled has been found to be very low under certain experimental conditions (Cope *et al* 1943 Reitemeier *et al* 1957 Moll and Code 1962)

In the present study the net fluxes of sodium and chloride ions on intragastric instillation of NaCl solutions of various concentration were examined Because the equilibrium concentration of sodium ions in the resting stomach is about 140—150 mM (see *e g* Linde and Öbrink 1950) one would expect that no net flux of sodium ions would occur on instillation of about 150 mM NaCl From Fig 5 it may be estimated however that about 100 $\mu$Eq/30 min would enter the stomach on instillation of 155 mM NaCl. This figure compares well with the amount of sodium ions that entered the stomachs in the 30 min preceding the first HCl instillation (Table II) Provided that the influx of sodium ions as an effect of secretion was the same on instillation of distilled water and on instillation of 310 mM NaCl and also that the mucosal ion permeability did not differ in either case from the control value the expected relationship between the net flux of sodium ions and the mean sodium ion concentration in the instillate in the absence of a sodium ion containing basal secretion is shown by the interrupted line in Fig 5

One factor that in addition to the NaCl containing basal secretion might partly explain the small net efflux of NaCl on instillation of 310 mM NaCl is that the mucosal ion permeability decreased on instillation of moderately hyperosmotic NaCl solution This effect of application of moderately hyperosmotic solutions on the mucosal side of gallbladder epithelium *in vitro* (the DC resistance increased) has been reported by Smulders *et al* (1972) It is not known whether the same occurs in organs with the circulation intact, however If this were the case the slope of the proportionality line in Fig 5 would be somewhat steeper but this would not affect the conclusions presented here.

On instillation of very hyperosmotic NaCl solutions the net effluxes of sodium ions were larger than expected from an increase in proportion to the mean concentration difference between the gastric lumen and the blood (see Fig 5) It should be noted (as has been observed earlier by *e g* Teorell 1933 Rehm *et al* 1953 Altamirano 1969 Moody and Durbin 1969) that fluid moved into the stomach on instillation of hyperosmotic solutions Regardless of whether the fluid consisted of water and electrolytes moved osmotically from the mucosa (*cf* Altamirano 1969) or whether it was secretion that contained NaCl (*cf* Rehm *et al* 1953) the net efflux of NaCl from the stomach would be reduced The conclusion drawn is that on instillation of highly concentrated NaCl solutions the mucosal ion permeability increased *From the result it cannot be determined whether the increased net efflux* of NaCl took place transcellularly or was due to decreased extracellular resistance to ion fluxes or to both. On examination in the scanning electron microscope (SEM) of gastric mucosae exposed to 1550 mM NaCl for 30 min and then fixed

either in roughly iso-osmotic fixation solutions or in fixation solutions made hyper osmotic by adding NaCl intercellularly located round openings were observed on the mucosal surface (Frenning 1973), see Fig 6 It is probable that NaCl moves into the cells on instillation of very hyperosmotic NaCl solution into the stomach but the observations in the SEM suggest that a decreased extracellular resistance to ion fluxes might be the main explanation for the increased net efflux of NaCl

On instillation of 170 mM HCl subsequent to an instillation of distilled water or of 310 or 620 mM NaCl there were only minor differences in the mean net effluxes of hydrogen ions in comparison with the control values The mean net influxes of sodium ions however were increased in all HCl instillation periods following an instillation of 620 mM NaCl and in the first period following an instillation of 310 mM NaCl In these periods there were also mean net influxes of chloride ions In the first instillation period following an instillation of NaCl residual amounts of NaCl in the mucosa probably influenced the results though the stomachs were rinsed with HCl before this period was begun It is probable that part of the NaCl absorbed in the preceding period moved back into the lumen as a consequence of reversal of the diffusion gradient It is less likely however that this situation would persist throughout all instillation periods (*i e* also in $HCl_3$ and $HCl_4$) Yet in these periods the net influx of sodium ions remained increased The increased net influx of sodium ions might be explained by an increase in the electrolyte permeability of the gastric mucosa as suggested by Altamirano (1969) but a concomitant net gain of water and an unchanged efflux of hydrogen do not support such a view A net influx of chloride ions on instillation of 170 mM HCl is difficult to explain if there was no secretion or no transudation Of the e two possibilities transudation appears unlikely because if the permeability of the mucosa was increased to such an extent that transudation occurred one would also expect that an increased amount of hydrogen ions would move out of the stomach furthermore a transudate would contain bicarbonate which would neutralize acid and manifest itself as an increase in the net efflux of hydrogen ions Nor does the fact that the surface morphology of gastric mucosa exposed to 620 mM NaCl was essentially normal support this view (Frenning 1973) A secretion of a NaCl containing fluid to an extent shown by the oncrease in volume thus appears to be the most probable explanation for the results (*cf* Rehm *et al* 1953 Frenning 1972 a and b)

There was no increase in the net efflux of hydrogen ions on instillation of 170 mM HCl subsequent to an instillation of up to 620 mM NaCl Subsequent to an instillation of 930 or 1550 mM NaCl the net effluxes of hydrogen ions on instilla tion of HCl were increased It would be difficult to explain this result except as an effect of an increa ed gastric mucosal ion permeability On gastric mucosae exposed first to 1550 mM NaCl and then to 170 mM HCl and fixed thereafter cellular swelling and cell disruption were observed in the SEM (Frenning 1973)

DC resistance measurements on isolated cells and on isolated gastric mucosae led to the conclusion that extracellular low resistance shunts exist in the normal in tact ga tric mucosa (Blum *et al* 1971) It is not improbable that the electrolyte

transport observed on instillation of 170 mM HCl in normal stomachs takes place in these shunts

Subsequent to exposure of the gastric mucosa to certain weak acids its ion permeability increases and this is believed to be due to increased extracellular migration of electrolytes (Frenning 1972 b, Frenning and Öbrink 1971 Flemstrom 1971 b) In the present work it was found that relatively large osmolality variations in the gastric lumen were needed to affect the gastric mucosal ion permeability It is believed that the increased loss of hydrogen ions on instillation of 170 mM HCl subsequent to an instillation of 930 or 1550 mM NaCl was due mainly to an increased efflux of hydrogen ions between swollen cells and through membrane residues of disrupted cells (see Frenning 1973) As regards the increased net influx of sodium ions in these experiments it is probable that in addition to the sodium entering as NaCl containing secretion (*cf* above) part of the increment was a consequence of increased diffusional transport via the same pathways through which the hydrogen ions disappeared from the stomach In these expts it appears likely that transudation also occurred

Application of hyperosmotic NaCl solution (1200—2000 mM) on the gastric mucosa has been reported to decrease the output of acid from the stomach (Rehm *et al* 1953 Kowalevski *et al* 1970) In view of the present results this can be explained at least partly as an effect of increased gastric mucosal permeability to electrolytes (*cf* Teorell 1947 Öbrink 1948 Nordgren 1963)

*In conclusion* The gastric mucosa resisted exposure to NaCl solution up to 4 times as concentrated as physiological saline without any detectable effects on its ion permeability Production of a NaCl containing fluid was noted subsequent to an instillation of 620 mM NaCl but more concentrated NaCl was needed to increase the electrolyte permeability of the gastric mucosa both on instillation of the NaCl itself and during subsequent HCl instillations

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# Reflex Adrenergic Inhibition of Gastric Motility Elicited from the Gastric Antrum

By

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## Abstract

ABRAHAMSSON H *Reflex adrenergic inhibition of gastric motility elicited from the gastric antrum* Acta physiol scand 1974 *90* 14—24

The reflex effects of distension and contraction of the gastric antrum on corpus fundus motility were studied in anesthetized cats Besides a vagally mediated reflex corpus fundus relaxation antral distension elicited a prompt reflex inhibition of corpus-fundus contractions that was present also after vagotomy In vagotomized animals this inhibitory reflex persisted also after cervical spinal cord transection but was abolished by spinal anesthesia or by guanethidine Stepwise increases of antral distension revealed that the reflex corpus fundus inhibition appeared already at distending pressures of 5—*10* cm $H_2O$ then increased in a graded fashion and could be maintained for at least 10 min if a steady distension was applied. Also isometric antral contractions were effective in eliciting the reflex corpus-fundus inhibition — It is concluded that activation of slowly adapting tension receptors in the antral gastric wall inhibit gastric (corpus fundus) motility *via* a spinal reflex mechanism mediated by splanchnic adrenergic fibres that seem to suppress the intramural excitatory cholinergic neurons This gastro-gastric adrenergic inhibitory reflex seems to be activated concomitantly with a vago-vagal gastro-gastric relaxatory reflex It is suggested that these two suppressive reflex mechanisms play an important role in regulation of gastric motor functions

In a previous report (Abrahamsson 1973) the effect of distension of the gastric antrum on the motility in the corpus fundus part of the stomach was studied Antral distension was found to produce corpus fundus relaxation by reflex activation of the vagal non adrenergic relaxatory nerve fibres to the stomach It was then also observed that when rhythmic corpus fundus contractions were present antral distension could inhibit these contractions as well, even if vagal nervous activity was abolished

In the present experiments this non vagal inhibitory reflex effect of antral distension on corpus fundus motility is analysed with special reference to receptors and nervous mechanisms involved Attention was directed to the fact that if sympathetic mechanisms proved to be involved one must consider the adrenergic fibres to exert their inhibitory gastric effect mainly on intramural cholinergic excitatory neurons (Jansson and Martinson 1966)

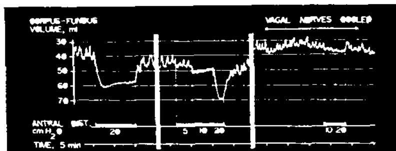



Fig 1 Cat 3 2 kg Suppressive effects on corpus-fundus motility in response to graded distensions of the gastric antrum *Left panel* A long lasting antral distension (pressure head 20 cm $H_2O$) produces both a maintained pronounced volume increase and an inhibition of rhythmic contractile activity in corpus-fundus *Middle panel* Upon stepwise increases of antral distension both the mentioned types of inhibitory responses in corpus fundus appear at a distending pressure of 10 cm $H_2O$ and are very pronounced at 20 cm $H_2O$ *Right panel* During cold blockade of the vagal nerves antral distension still produces inhibition of corpus fundus contractions but the pronounced volume increase seen in the left and middle panels is now abolished

## Material and methods

30 cats weighing 2 2—4 9 kg and deprived of food for 24—36 h were used for the experiments 6 of the animals were also used in a previously reported study (Abrahamsson 19 3) After induction with ether they were anesthetized with chloralose 50—60 mg/kg b w and a tracheal cannula was inserted

In 3 cats gastric motility was recorded as volume changes at a low pressure (Jansson 1969 a) by means of a large gastric balloon inserted *via* the esophagus and connected to a water filled reservoir In the remaining 27 cats an abdominal wall incision was made and 2 balloons were inserted *via* the esophagus and placed in the gastric antrum and corpus-fundus respectively their positions being secured by ligatures as described previously (Abrahamsson 1973) The corpus-fundus balloon was connected to a water filled reservoir either connected to a float recorder or weighed by a force transducer operating a Rikadenki writer Thereby corpus fundus motility was recorded as volume changes at a low pressure 4—10 cm $H_2O$

The antral balloon was connected to an adjustable water filled reservoir which was either connected to a float recorder or weighed by a force transducer for recording of the volume of the antral balloon By adjusting this reservoir graded isotonic antral distensions could be performed at pressure heads of 1—40 cm $H_2O$ The catheter to the antral balloon was also connected to a Statham pressure transducer (P23AC) coupled to the Rikadenki writer Isometric antral distensions *i e* when antral contractions were allowed to act on a fixed volume in the antral balloon were made by first applying a slight isotonic antral distension after which the catheter to the distending pressure head reservoir was clamped

Vagal nervous activity was blocked at the cervical level either by local cooling with metal tubes perfused with a mixture of ice water and alcohol or by nerve section To induce excitatory gastric motor activity electric stimulation of the distal ends of the cut vagal nerves were made by bipolar silver electrodes Stimulation parameters were 0 5—16 Hz 0 2—4 ms and 4—10 V In 6 expts electric stimulation of the central end of cut antral nerve branches were performed at 1—40 Hz 1—5 ms and 5—10 V

In 2 expts transection of the spinal cord was performed at the C 6 level and in 5 expts spinal anesthesia was induced at the lumbar and thoracic levels by subdural administration of 2—4 ml of 2 % lidocain solution (Xylocain® Astra) through a fine polyethylene catheter

Guanethidine (Ismelin® CIBA) was administered i v in a dose of 3—5 mg/kg b w and atropine (Atropine sulphate Merck) in a dose of 0 5—1 mg/kg b w i v Artificial respiration was maintained by a respiration pump in the spinal preparations Blood pressure was measured by a mercury manometer or by a pressure transducer connected to a femoral artery

## Results

### I *Suppressive effects of antral distension on corpus fundus motility*

Distension of the gastric antrum when performed during periods of rhythmic corpus fundus activity produced within 5—7 s two different effects on corpus-fundus

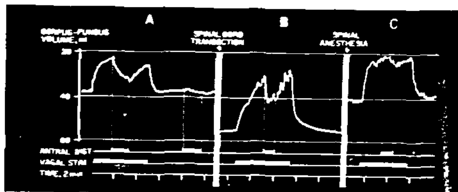



Fig 2 Cat 2 2 kg vagotomized. *A* Inhibition of a vagally induced corpus-fundus contraction by antral distension No effect of antral distension is obtained when corpus-fundus contractions are absent. *B* An inhibitory effect of antral distension persists after cervical (C6) transection of the spinal cord. *C* The inhibitory effect of antral distension is eliminated when the spinal cord is anesthetized by lidocaine. Efferent vagal stimulation at 1 Hz, 3 ms and 8 V Antral distending pressure head 25 cm $H_2O$

motility One effect was a pronounced increase of corpus-fundus volume (Fig 1 left and middle panel a relaxatory response which was abolished by vagal blockade (Fig 1 right panel) as also described in detail earlier (Abrahamsson 1973) The other response was a prompt inhibition of prevailing rhythmic activity In contrast to the mentioned vagally mediated corpus-fundus relaxation this type of inhibition of rhythmic activity as a result of antral distension was obtained also after vagal blockade (Fig 1 right panel) provided rhythmic activity was then still present If however vagotomy largely abolished these rhythmic corpus-fundus contractions an antral distension had no or only a slight effect (see below) but its pronounced inhibitory effect reappeared if corpus-fundus contractions were induced by direct vagal stimulation Fig 2 panel A) This circumstance was utilized in the further analysis of this non vagal inhibitory reflex mechanism

## *II Analysis of the non vagal inhibition of corpus fundus motility induced by antral distension*

*Antral distension produced as shown in Fig 2 a prompt inhibition of the corpus-*fundus contractions that were induced by activation of the excitatory vagal fibres. This inhibitory response persisted after cervical transection of the spinal cord (Fig 2 panel B but was eliminated by spinal anesthesia (Fig 2 panel C) as well as by blockade of the splanchnic nerves Further selective exclusion of the adrenal gland did not influence this inhibitory reflex response but it was completely abolished by guanethidine Fig 3 right panel These results show that the inhibitory corpus-fundus response to antral distension is due to a spinal reflex mediated *via* non vagal presumably splanchnic afferents and efferent splanchnic adrenergic fibres to the stomach.

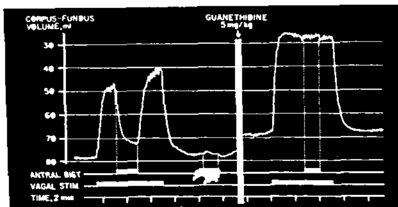



Fig 3 Cat ?8 kg vagotomized Antral distension elicits pronounced inhibition of vagally induced corpus fundus contraction the inhibitory response to antral distension is completely abolished by guanethidine 5 mg/kg iv vagal stimulation 3 Hz 2 ms 8 V Antral distending pressure 25 cm $H_2O$

The stimulus response characteristics for the described inhibitory adrenergic reflex were studied in 6 cats by graded increases of isotonic antral distension (Fig 4 Fig 1 middle and right panel) The threshold distending pressure for elicitation of the reflex response was throughout below 10 cm H O and sometimes an antral distending pressure of only 5—6 cm H O was sufficient to elicit clearcut reflex effects The degree of corpus fundus inhibition increased with increasing antral distension as illustrated in Fig 4 and pressures of 20 cm H O always elicited pronounced inhibition Further Fig 1 (left panel) and 5 illustrate that a sustained inhibition of corpus fundus activity occurs when antral distension at a pressure of 20 cm H O is maintained for about 10 min Such maintained inhibitory responses were observed both in vagotomized and vagally intact preparations provided they exhibited rhythmic corpus fundus contractions Maintained corpus fundus inhibition occurred whether

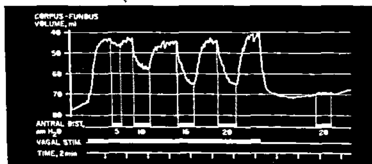



Fig 4 Cat ?8 kg vagotomized Inhibitory effect of graded antral distensions (pressure head increased from 5 to 20 cm H O) on vagally induced corpus fundus contractions No effect of antral distension is seen when corpus fundus contractions are absent (to the right) Vagal timulation at 3 Hz 2 ms 8 V

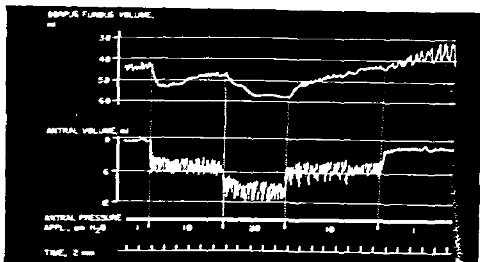



Fig. 5. Cat 3.2 kg. Vagal nerves intact. Corpus-fundus and antral responses to long-lasting stepwise increase and decrease in antral distension (pressure changed from 1 to 20 cm $H_2O$). Note the decrease of corpus-fundus volume and the reappearance of rhythmic corpus-fundus contractions when antral distension is again decreased. The rhythmic antral contractions are still pronounced when the antral distension pressure is raised from 10 to 20 cm $H_2O$.

antral volume changes were present (Fig. 5) or not during the distension. When after a prolonged steady antral distension the antral pressure was decreased to a lower level (Fig. 5) there was a corresponding increase in corpus-fundus motility indicating a graded release of inhibitory reflex influence.

In some experiments the stomach was inspected directly through the abdominal incision. It was observed that efferent vagal stimulation produced intense peristaltic contractions propagating along the corpus in aborad direction. Antral distension immediately widened the corpus-fundus part and reduced or even largely abolished the peristaltic contractions. When a sustained antral distension at a pressure of 15–20 cm $H_2O$ was maintained the peristaltic corpus contractions remained suppressed throughout the distension period only to reappear in full extent as soon as the antral distension was interrupted.

As mentioned above antral distension induced inhibitory responses in the corpus-fundus after vagotomy only when the corpus-fundus region still exhibited rhythmic contractions. If no such contractions were present antral distension was in most instances without effect (Fig. 2A, Fig. 3 left panel, Fig. 4) whether the prevailing gastric tone was high or low. Sometimes however a slight slowly developing volume increase was recorded in response to intense antral distension but first after a latency of at least 30 s; after administration of atropine the latency for this minor response was 40–60 s. However also this slight delayed inhibitory response was abolished by guanethidine. All the mentioned observations are in accordance with the concept that the splanchnic adrenergic fibres to the stomach exert their inhibitory effect on gastric motility mainly by acting on intramural cholinergic neurons (Jansson and Martinson 1966, Jansson 1969 b).

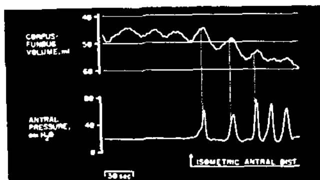



Fig 6 Cat 4 9 kg vagotomized Inhibitory effect of isometric antral contractions on corpus fundus motility induced by continuous stimulation of the vagal excitatory fibres. When the isotonic antral distension to the left (pressure kept at 20 cm $H_2O$) is switched to an isometric distension the induced antral pressure waves are within 4—6 s followed by corpus fundus inhibitions Continuous vagal stimulation at 4 Hz 3 ms and 3 V Pressure recording in this figure and Fig 7 made by a side arm of the catheter connecting the pressure reservoir with the antral balloon

While the antral distension markedly inhibited prevailing corpus fundus contractions rhythmic contractions in the distended antrum itself continued apparently unchanged or were even enhanced This is illustrated in Fig 5 where the antral contractions are still pronounced when antral distending pressure is raised from 10 to 20 cm $H_2O$ while this procedure greatly reduced corpus fundus activity

*III Comparison of effects of isotonic and isometric antral distension*

In the abovementioned analysis of the adrenergic inhibitory reflex from the gastric antrum to the corpus fundus part graded isotonic distensions of the antrum at a fixed pressure load were usually employed However to gain further information about the receptor mechanism involved isometric antral distension *i e* at fixed volumes were also performed and the reflex effects were compared with those produced by isotonic antral distensions A characteristic finding is illustrated in Fig 6 showing a sequence in which a steady isotonic antral distension (pressure 20 cm $H_2O$) is changed to an isometric one At the first isometric antral pressure wave an increase of corpus fundus volume appears within 4 s The subsequent antral contractions are also with a latency of 4—6 s accompanied by further increases in corpus fundus volume despite the fact that the antral volume remains constant

When isotonic antral distensions were repeatedly changed to isometric ones as in Fig 6 the time between the onset of the isometric condition and the first appearing isometric antral contraction varied from 1 to about 12 s However independent of this latency the induced corpus fundus inhibition invariably appeared 4—6 s after the upstroke of the first appearing antral pressure wave indicating that the reflex inhibitions were closely related to the antral contractions and not induced by the isometric condition *per se*

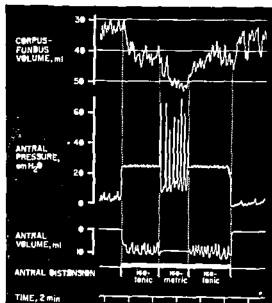



Fig 7 Cat 2 5 kg vagotomized Effect of isotonic and isometric antral distension on corpus fundus motility induced by a continuous stimulation of the vagal excitatory fibres The isotonic antral distension (pressure 25 cm H O) leads to a prompt reflex corpus fundus inhibition After switching to isometric antral distension a further corpus fundus inhibition occurs immediately after the first antral pressure wave and is maintained during the period of isometric antral contractions Switching from isometric to isotonic antral distension is followed by a regain of corpus fundus motility further accentuated when the antral distension is abolished Note that the mean antral volume is essentially the same during the isometric and the second isotonic antral distension Vagal nerves stimulated throughout the recording at 4 Hz 3 ms 6 V

The problem is further elucidated in Fig 7 where an efferent vagal stimulation is performed throughout An isotonic antral distension at a pressure of 25 cm H O immediately leads to inhibition of corpus fundus motility and during this isotonic antral distension rhythmic antral volume changes appear When the distension is changed to an isometric one the appearing antral isometric contractions are accompanied by an accentuated corpus fundus inhibition remaining throughout the isometric antral distension When the antral distension is again changed to an isotonic one a clearcut decrease in the corpus fundus inhibition is seen despite the same average antral volume When in turn this isotonic antral distension is finished *the corpus fundus activity returns towards control indicating that a prevailing in*hibitory influence is now eliminated

*IV Afferent electric stimulation of antral nerves*

In six vagotomized cats studies were performed concerning the effect of afferent electric stimulation of antral nerve branches on vagally induced gastric contractions Such stimulations produced within 4—5 s an inhibition of gastric contractile activity in the same manner as described above for antral distension Already frequencies of 5—10 Hz produced pronounced inhibitory reflex effects and little further inhibition was gained by still higher rates Also these inhibitory effects were abolished by spinal anesthesia and by administration of guanethidine Therefore also these results show that activation of antral afferents can produce inhibition of gastric contractile activity by a spinal gastro gastric reflex mechanism presumably involving splanchnic afferents and efferent splanchnic adrenergic fibres

## Discussion

From the present results it is evident that activation of mechanoreceptors in the gastric antrum inhibits motility in the proximal "reservoir part" of the stomach by a reflex activation of splanchnic adrenergic fibers to the stomach. This non-vagal inhibitory gastro-gastric reflex, organized at a strictly spinal level, seems to be activated in close parallel with the vago-vagal non-adrenergic relaxatory reflex to the stomach that is also elicited by mechanical antral stimulation (Abrahamsson 1973). Thus the reservoir part of the stomach is under suppressive influence by two distinctly separate extrinsic reflex mechanisms operated by antral mechanoreceptors.

The results indicate that the afferent link of the non-vagal inhibitory reflex is constituted by antral mechanoreceptors with afferents in the splanchnic nerves. In contrast to the relatively well known gastric mechanoreceptors with vagal afferents (see Leek 1971), few data are available concerning gastric receptors with splanchnic nerve afferents. Reports of reflex effects of gastric distension on duodenal motility (Daniel and Wiebe 1966) and of abomasal distension on movements in the ruminant reticulum (Titchen 1958) suggest, however, that gastric mechanoreceptors with splanchnic nerve afferents exist. Their role in regulation of gastric motility in non-ruminants has so far not been clarified.

The reflex non-vagal inhibition of corpus-fundus motility was induced both by passive antral distension and by "isometric" antral contractions, i.e. this inhibitory reflex can apparently also be activated by mere increases in wall tension. The reflex response could be maintained for at least 10 min if a steady antral distension was applied. The gastro-intestinal receptors with splanchnic nerve afferents studied so far have not been shown to signal such mechanical events. From studies of splanchnic afferents with the "single fibre" technique Morrison (1972) reported the presence of mechanoreceptors in the mesentery close to the intestine exhibiting a rapidly adapting discharge in response to intestinal distension and a maintained discharge during stretch of the mesentery. The reflex response to antral distension in the present study differs from the discharge of these receptors to intestinal distension because the reflex response could be maintained for long periods whether the antral distension was isometric or isotonic. The present results hardly suggest that "movement receptors" (Bessou and Perl 1966) are responsible for the reflex effects, since these were more pronounced with isometric than with isotonic antral distension (Fig. 7), although in the latter case antral movements are likely to have been more pronounced. Therefore, a reasonable explanation at present seems to be that the antral receptors inducing the described gastro-gastric adrenergic inhibitory reflex are constituted by slowly adapting "tension receptors", similar to those with vagal afferents (for ref. see Leek 1971). This type of receptors responds to increases of tangential wall tension, which would agree well also with the characteristics of the receptor mechanism eliciting an antro-gastric adrenergic inhibitory reflex.

The results from cervical spinal cord transection, spinal anesthesia and splanchnic nerve blockade show that the gastro-gastric adrenergic inhibitory reflex is dependent on intact connections with the spinal cord but does not require the participation of

supra pinal structures. In this respect the presently described reflex mechanism is similar to the intestino-gastric adrenergic inhibitory reflex (Jansson and Martinson 1966) and the intestino-intestinal inhibitory reflex (Johansson and Langston 1964) which are considered to be true spinal reflexes. The fact that the gastro-gastric adrenergic inhibitory reflex persisted after cervical spinal cord transection does of course not exclude that supraspinal structures can modify this spinal reflex (see e.g. Johansson, Jonsson and Ljung 1965).

Throughout the present experiments antral distension in vagotomized preparations elicited its clearcut inhibitory action whenever corpus fundus contractions were present, whether these occurred "spontaneously" or were induced by efferent vagal stimulation. However, in the absence of such contractions the inhibitory corpus-fundus response to antral distension was very scant and had a latency of 30 s or more. These results are fully in accordance with the present concept that the adrenergic fibres exert their essential inhibitory influence on gastro-intestinal motility by an action on intramural excitatory neurons. Strong morphological and physiological evidence for such an arrangement has been presented (e.g. Norberg 1964, Jacobowitz 1965, Jansson and Martinson 1966, Jansson 1969 b, Cabella 1971).

The results presented here, together with those earlier reported (Abrahamsson 1973), indicate that the gastro-gastric adrenergic inhibitory reflex to corpus fundus and the vago-vagal gastric relaxatory reflex, both emanating from antral mechanoreceptors, exert their suppressive influences on gastric motility in close parallel under physiological circumstances. Thus the two reflex mechanisms seem to have about the same low threshold for elicitation. Both reflexes are elicited from a slowly adapting receptor mechanism activated by antral distension and by antral contraction, i.e. the receptors for both reflexes seem to be tension receptors. However, the afferent fibres take different courses in the vagal and splanchnic nerves, respectively. Furthermore the efferent links of the two reflex mechanisms show some clear differences which are inherent in the differences in mode of action of the vagal non-adrenergic relaxatory fibres and the splanchnic adrenergic fibres, respectively (cf. Jansson 1969 b). Thus the gastro-gastric adrenergic inhibitory reflex can suppress tonic and phasic corpus fundus activities to the extent that these activities are initiated by intramural cholinergic neurons, according to the concept of a ganglionic site of action for the adrenergic fibres in the gastric wall. The vago-vagal relaxatory reflex, on the other hand, can greatly modify smooth muscle tone of the corpus fundus part independently of the activity of the intramural excitatory neurons. Thus the two separate reflex mechanisms can evidently supplement each other by their different mode of action on the effector organ, thus promptly producing a more effective suppression of the corpus fundus motility than when activated separately.

The mechanical events activating the two reflexes indicate that they may both be involved in the regulation of gastric adaptation to increasing volumes (gastric receptive relaxation) which is an important feature in gastric reservoir function. In this respect evidence for the importance of vago-vagal gastro-gastric relaxatory reflexes has already been presented (Abrahamsson and Jansson 1973, Abrahamsson

1973) but it seems likely that also the splanchnic adrenergic reflex mechanism may be involved in the regulation of the gastric reservoir capacity. It is, furthermore likely that the two inhibitory reflex mechanisms induced from the antrum to affect corpus-fundus activity and volume constitute a feedback regulation of the net transport of gastric contents from the reservoir part to the antral pump. The tone of the corpus-fundus reservoir will influence the force whereby gastric contents are moved into the antrum. Whenever the tone of the reservoir is raised, the bulk subjected to compression by the antral pump will increase which, in turns, leads to increased antral tension. This will, via the described inhibitory reflexes from the antrum, suppress corpus-fundus activity and so on. Such a type of regulatory mechanism would keep the tone of the gastric reservoir at a suitable level to give a proper aborad pressure gradient within the stomach and hence influence gastric emptying. Marked rhythmic rises in antral pressure and thus also in antral wall tension occur in the stomach digesting solid food (Thomas 1957) and solid food is slowly emptied from the stomach.

From the above description of the organization of gastro-gastric vagal and splanchnical inhibitory reflexes it follows that it may be difficult to draw conclusions from effects of e.g. sectioning of the splanchnic nerves on gastric motility. An abolishment of the gastro-gastric adrenergic inhibitory reflex by such a procedure may to some extent be compensated for by an increased engagement of the vago-vagal gastro-gastric relaxatory reflex mechanism. Studying the gastric pressure response at various levels of gastric filling in the cat, Perret and Hesser (1960) observed a much more pronounced pressure response after combined sympatho-splanchnicotomy and vagotomy than after vagotomy alone. This is in accordance with the arrangement of gastro-gastric inhibitory reflexes, as outlined above, if the gastric tone after vagotomy is dependent on activity of intramural excitatory neurons (cf. Jansson and Lisander 1969).

The present results suggest that antral mechanoreceptors by their strategic position, by signalling the proper mechanical events, and by mediating appropriate reflex responses via two different but synergistic mechanisms, are well suited to play an important role in the control of various gastric motor functions. As far as extrinsic nervous reflexes are concerned both sets of inhibitory nerve supplies to the stomach, the vagal non adrenergic relaxatory fibres and the splanchnic adrenergic inhibitory fibres, are involved in this regulatory system.

*Added in proof.* Morrison (personal communication) has recently found gastric mechanoreceptors, partly located beneath the serosa and with afferents in the splanchnic nerves, giving a slowly adapting discharge to antral distension and activated also by antral contraction.

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# Response of Isolated Frog Muscle Spindle to Sine Wave Stimulation

By

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## Abstract

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The impulse response and the receptor potential of the isolated muscle spindle of the frog were recorded during sinusoidal stretching at frequencies from 0 1 c/s to 100 c/s Attention was focused on the changes of the responses in the initial phase of stimulation before the steady state was reached In the course of sine wave stimulation the impulse response of the spindle under goes regular changes characterized by an increase in threshold and latency of the individual impulses a decrease in phase lead and peak frequency and a reduction in number of spikes in each cycle The changes are most pronounced during the first 2—5 cycles and a steady state with a fixed impulse patterning is usually reached after 10—15 cycles The receptor potential evoked by a sine wave stretch is distorted in comparison with the waveform of the stimulus in having a relatively faster rise and slower decay With changes in frequency and amplitude of stimulation it undergoes characteristic alterations in phase lead and configuration When super imposed upon a constant stretch or a linearly rising stretch sinusoidal stimulation produces responses which increase in amplitude with the underlying stretch These observations provide evidence that the sensory endings responsible for the transducer action do not obey linear trans fer functions

Sinusoidal stimulation has been proved to be a useful method for analyzing various functional properties of the muscle spindle Attention has generally been focused on the response characteristics in a steady state after the spindle has been stimulated for a given period of time Recent studies by Kirkwood (1972) on the *in situ* spindle of the frog have provided important contribution to the knowledge of the behaviour of the spindle during sinusoidal length changes In the case of the mammalian spindle a number of studies (Lippold *et al* 1958 Stuart *et al* 1965 Lennerstrand and Thoden 1968 Poppele and Terzuolo 1968 Matthews and Stein 1969 Poppele and Chen 1972) have been reported on the linearities and non linearities of the cat spindle From data obtained in such studies the transfer functions for the spindle response have been derived (Poppele and Bowman 1970) Before the steady state is reached the response undergoes a progressive change in impulse pattern invol

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alterations in phase lead, peak frequency, latency, threshold and other response characteristics. Comparatively little interest has been paid to these changes and the mechanisms for their production is unknown.

The purpose of the present study was to analyze the changes of the impulse response of the muscle spindle in the early phase of sinusoidal stimulation and to examine how these changes were related to the properties of the underlying receptor potential. Another aim of the study was to find out whether or not the non-linear behaviour of the spindle (Matthews and Stein 1969) could be accounted for by the transducer action of the endings.

## Methods

All experiments were carried out on spindles isolated from m. ext. dig. long. IV of the frog *Rana temporaria*. The method for isolation, stretching and recording was essentially the same as described earlier (Ottoson, McReynolds and Shepherd 1969). The spindle was mounted in an experimental chamber and attached at resting length at each end to a nylon rod. One of the rods was fixed and the other was attached to the coil of a loud speaker. Stretch was applied by driving the loud speaker with sinusoidal pulses from a wave generator (GR13[illegible]). The movements of the pulling rod were monitored on the oscilloscope by use of a high-sensitive capacitance meter (Haapanen 1962).

*Recordings.* The afferent impulse discharge was recorded from the sensory nerve which was lifted up in the oil covering the Ringer solution in the experimental chamber. The nerve was connected to a calomel half-cell electrode by an agar filled fine tube. Another calomel half-cell electrode was connected to the solution in the chamber. The recorded signals were fed into a direct coupled amplifier and displayed on the screen of the oscilloscope. The recordings were also stored on magnetic tape from which the impulse response could be fed into a circuit which gave a display of the instantaneous discharge (Matthews 1963). In recordings of the responses to trains of sine wave stretches stationary beams of the oscilloscope were photographed on film moving at 100–250 mm/s. Following a train of stretches the spindle was allowed to rest for 30–60 s before stretch was again applied. To isolate the receptor potential the preparation was treated with lignocaine (0.18 [illegible]). To control that no movement artefacts were involved the spindle was crushed at the end of each experiment and test stretches applied (cf. Ottoson 1969).

*Solution.* The Ringer solution used had the following composition: NaCl 115 mM, KCl 2.5 mM, $CaCl_2$ 1.8 mM, $NaH_2PO_4$ 0.85 mM, $Na_2HPO_4$ [illegible] mM (pH [illegible]). All experiments were carried out at a temperature of 18 °C.

*Measurement of phase angle.* Accurate measurements of phase angles were difficult to achieve. For the receptor potential this was partly due to the low signal-to-noise ratio at low frequencies and amplitudes of stretch and to high frequencies and amplitudes to the time dependent non-linearity of the response. For the impulse response the uncertainties were much due to the fact that firing often occurred only during a small part of the cycle. To reduce the measurements error a family of parabolas of different focal lengths was constructed. The phase angle was determined by superimposing a suitable parabola onto enlarged pictures of the response for the best fit and using the symmetry axis of the parabola as an approximation.

## Results

### I. *General properties of the spindle response to sine wave stimulation*

#### a) *Impulse response*

The records in Fig. 1 illustrate the general characteristics of the spindle response to 3 different frequencies of sine wave stimulation. Since the spindle was mounted at resting length there was no carrier frequency of firing to be modulated and the response amplitudes during stretching were consequently relatively low. The records

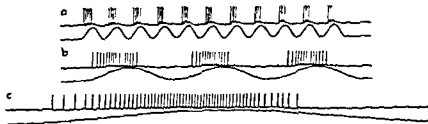



Fig 1 General characteristics of impulse response of muscle spindle to sinusoidal stretching at ? c/s (*a*) 1 c/s (*b*) and 0 1 c/s (*c*) Stretch shown on lower trace

show that for any given cycle the firing frequency increases with stretch up to a maximum value and then decreases before the stimulus has reached its peak amplitude This characteristic phase lead is more clearly brought out when the impulse response is displayed as the instantaneous impulse frequency (Fig 2) A close analysis of the relation between the response and the stimulus frequencies shows that for a given amplitude of stretch the peak frequency reached during the first cycle in a train increases with increasing frequency (Fig 3 *A*) For frequencies below about 1 c/s the response changes relatively little in amplitude with frequency while there is a rapid increase for frequencies higher than 2 c/s It is interesting to note the close similarity between the behaviour of the isolated spindle and the spindle *in situ* As recently demonstrated by Kirkwood (1972) the sensitivity of the non isolated spindle of the *extensor longus dig II* muscle of the frog increases with stimulating frequencies up to 10 c/s and decreases with higher frequencies while the

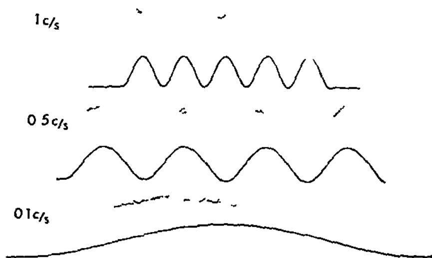



Fig 2 Frequency characteristics of response to sinusoidal stretch at 1 c/s 0 5 c/s and 0 1 c/s

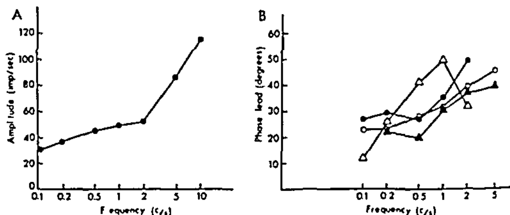



Fig. 3. *A* Response amplitude (peak frequency) in relation to frequency of stimulation. *B* Relation between phase lead and frequency of stimulation.

phase lead has a maximum at about 2 c/s. The frog spindle also exhibits certain similarities with the primary endings of the cat spindle (*cf.* Matthews and Stein 1969) as pointed out by Kirkwood (1972).

An analysis of the threshold for the impulses at different frequencies brings out several interesting features. As shown in the diagram in Fig. 4 A the stimulus threshold for the first impulses does not increase with stimulation frequency. This finding is in close agreement with earlier observations with linearly rising stretches (Shepherd and Ottoson 1965). For the following impulses there is an almost linear increase in threshold with stimulus frequency. It can also be seen that for a given frequency there is a decrease in the increment in threshold for the successive spikes in the impulse train whereas there is an increase in threshold increment with in

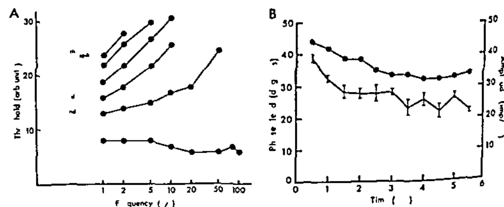



Fig. 4. *A* Threshold changes of individual impulses in response of spindle to different frequencies of sine wave stretch. *B* Time course of decrease in amplitude (filled circles) and phase lead of successive responses in train of sine wave stimulation at 2 c/s.

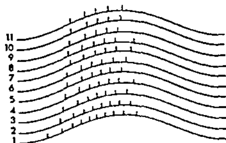



Fig 5 Schematic illustration of change in impulse pattern of successive responses (as indicated by numbers to the left) to train of sine wave stretching at 2 c/s

creasing stimulation frequency For instance at 50 c/s the threshold required to elicit the second impulse is more than two times higher than at 1 c/s These differences are to be attributed to the effect of refractoriness at the higher firing rates with increase in frequency of stimulation

The response characteristics described above apply for the first sine wave in a train For the following cycles there occurs a sequence of changes until a more or less steady state is reached Thus there is a gradual diminution in phase lead and a reduction of the amplitude of the response The time course of these changes is shown in the diagram in Fig 4 *B* The spindle was in this experiment stretched at a rate of 2/s for about 5 s Over this period the peak frequency decreased towards a constant value This change was accompanied by a corresponding decrease in phase lead The time constants for the changes of these two parameters of the response were closely related

In addition to the changes in phase lead and peak frequency the response undergoes a series of interesting alterations during the first 5—10 cycles of an applied train of sine wave stretches Some of these changes are illustrated by the tracings in Fig 5

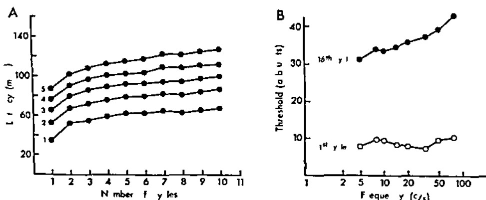



Fig 6 *A* Latency changes of first three spikes in response to train of sine wave stimulation at 2 c/s *B* Increase in threshold in course of train of sine wave stimulation Threshold values for onset of first spike in first and 16th cycle of response at different frequencies of sine wave stretching

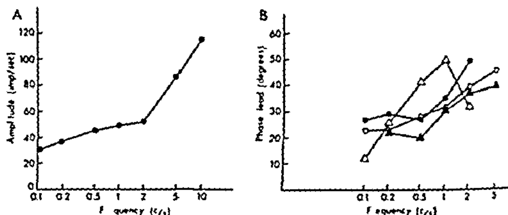



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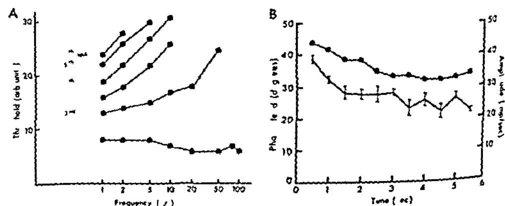



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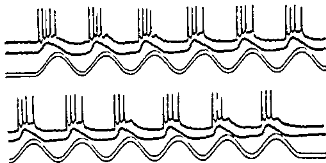


Fig 9 Receptor potentials and impulse responses to trains of sinusoidal stimulation at 5 c/s Photographically superimposed records of continuous recordings Bottom traces show stretch

and there are only minor changes with further prolongation of stimulation This state is usually reached after 10—15 cycles of stretch

The gradual delay for the appearance of the individual spikes of the impulse train over the first 10 cycles are brought out quantitatively in the diagram in Fig 6 A For each impulse the change is greatest over the first 5—6 cycles Following this the spikes more slowly approach their fixed time positions in the steady state A perhaps more interesting feature in the sequence of changes is that the curves for the time appearance of the individual spikes are more or less parallel It would appear that except for minor changes in time course the entire response is shifted towards a later phase of the sine wave stretch As a consequence of this the peak frequency of the response decreases to the value reached in the steady state This change can be seen by comparing the impulse response of the first and the 11th cycle of the recordings in Fig 5 In the first cycle the peak frequency is reached between the 5th and the 6th spike In the following cycles the whole response is shifted toward a later phase of the sine wave and in the 11th cycle the peak frequency is represented by the interval between the second and the third spike Although they appear at approximately the same phase angle as the 5th and the 6th spike in the first cycle the interval between them is approximately the same as that between the 2nd and 3rd spikes in the first cycle This change appears to account for the change in peak frequency as shown in Fig 4 *B* From this it should however not be concluded that the decrease in peak frequency is the consequence of a simple shift in time of the impulse response As will be discussed below a number of factors appear to contribute to the changes in time characteristics of the response

The shift of the impulse response towards a later phase of the sine wave stimulus reveals an increase in threshold for the generation of the spikes The change in threshold is quite considerable as is brought out in the diagram in Fig 6 *B* which shows the threshold changes for the first spike in the first and the 16th cycle in trains of sine wave stimulation at different frequencies As already described the threshold is approximately the same for the first cycle for the frequency range 5—100 c/s For the 16th cycle the threshold has increased 3—4 times and this change is more pronounced for the higher frequencies A possible explanation for this seems to be the contribution of accummulated refractoriness with higher frequencies of impulse firing

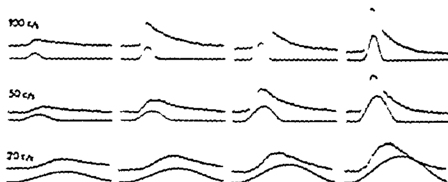



Fig 10 Receptor potentials to increasing amplitude of sinusoidal stretching

b *The receptor potential*

Above we have described the characteristics of the impulse response of the spindle to sinusoidal stretches and the changes it undergoes in the initial phase of stimulation It appeared important to find out how these features were related to the underlying receptor potential The depolarization of the endings is usually concealed by the superimposed impulse response and is therefore not easily studied unless the conducted activity is blocked Under certain experimental conditions such as at low amplitudes of relatively high frequency stimulation the graded response of the endings can however be obtained in isolation One example of this is shown by the records in Fig 7 The amplitude in this run was set just at threshold for evoking the impulse of the afferent fibre At 40 c/s only the first cycle of the sine wave stimulus evoked an impulse while the following cycles produced small wave like potentials With higher frequencies these potentials summated and produced spikes at regular intervals as the threshold for the fibre was reached It would appear that the records in Fig 7 reveal an important property of the endings When subjected to near threshold stimulation the refractoriness of the impulses elicited by the first cycle wipes out the impulse response for the following cycles This effect of refractoriness is overcome by the summation of the receptor potentials of the following cycles and in direct time relation to the frequency of the stimulus so that the afferent discharge rate is a multiple of the applied stimulus frequency A similar interaction between refractoriness and the impulse generating effect of the receptor potential occurs when the spindle is stimulated at a given frequency at or just above threshold as shown in Fig 7 *e*–*h* It seems likely that conditions of stimulation like this might occur in the spindle *in situ* for instance during fibrillation of the extrafusal fibres

More precise information about the relation between the characteristics of the impulse response and the underlying receptor potential was obtained by first recording the impulse response to sine wave stimulations at different frequencies and amplitudes and then recording the receptor potentials to the same stimulations after blocking of the impulse activity In this way a direct comparison could be made between various characteristics of the impulse response and the corresponding parameters of

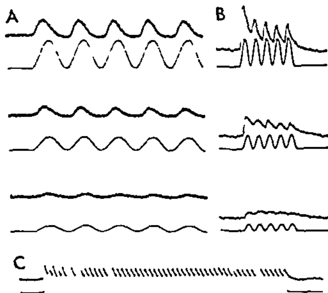



Fig 11 Summation of receptor potentials with increasing frequency of stimulation. Records in *A* and *B* show responses to 5 c/s and 100 c/s at three amplitudes of stretch. Record in *C* illustrates regularity of responses during prolonged stimulation at 50 c/s

the receptor potential The records in Fig 8 illustrate this approach It can be seen that the characteristics of the impulse response are closely related to the features of the underlying receptor potential In addition to this the records show that the prepotentials play an important role in the impulse patterning process As shown earlier (Ottoson and Shepherd 1970) there is a tight coupling between the receptor potential and the prepotentials This coupling ensures that the impulse response closely follows the transducer action A similar close relation also exists for the changes of the impulse response and the receptor potential in the course of a train of sine wave stretches as shown in Fig 9 In the following we will described in detail the behaviour of the receptor potential and its relation to the impulse response

The general behaviour of the receptor potential to increasing amplitudes of sine wave stretching at 100 50 and 20 c/s is illustrated in Fig 10 At low amplitudes of stretch the shape of the response appears to approach most closely the waveform of the stretch but does not exactly reproduce its time course In general the rising phase of the response is faster than the falling phase This characteristic feature becomes gradually more pronounced as the amplitude of the stretch is increased The distortion of the response shows that the input-output relation of the transducer action of the spindle is not linear

A characteristic feature of the receptor potential is that the individual responses in a train summate when the stimulus frequency is increased beyond a given value In *A* in Fig 11 a train of stretches at 5 c/s was applied At this frequency the individual responses are clearly separated for different amplitudes of stretch When the stimulus frequency goes beyond about 10 c/s summation begins to occur The configuration of the response at frequencies above 10 c/s is dependent on the amplitude as well as the frequency of stimulation An example of the effect of increasing amplitude at a given frequency is shown in Fig 11 *B* For low amplitudes a steady

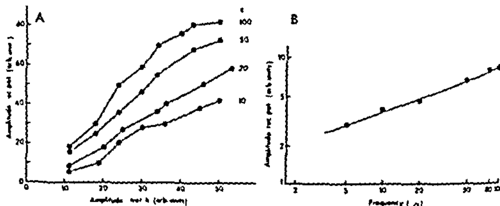



Fig. 12. A. Receptor potential amplitude of first cycle at different frequencies and amplitude of sine wave stretch. B. Change in amplitude of receptor potential (first cycle) with increase of frequency of sinusoidal stretch.

depolarization with small superimposed waves is built up. As stretch is made stronger each cycle of the stimulus produces a pronounced elevation. The peaks of these individual responses are superimposed upon a sustained depolarization and the peak amplitudes decrease towards a steady value with time. The response to prolonged stimulation is characterized by a high degree of regularity as seen in *C* in Fig. 11.

It has been shown in earlier studies with linearly rising stretches (Ottoson and Shepherd 1965) that the amplitude of the receptor potential increases in direct proportion with the amount of stretch. This is also true for sinusoidal stretches as is illustrated in Fig. 12 *A*. For a given frequency the response amplitude increases in direct proportion with the amplitude of stretch. With increasing frequency the curves become steeper as an indication of the velocity sensitive behaviour of the endings. This is also brought out by the diagram in Fig. 12 *B* which shows in a log log plot the relation between the amplitude of the response (first cycle) and the sine wave frequency. The linear relation between the transducer action and the frequency of the stimulus appears to explain the typical increase in impulse response with frequency as described above.

At low amplitudes of stretch accurate determinations of the phase lead of the response were difficult because of the low signal to noise ratio. Another complicating factor was that the shape of the receptor potential varies with the amplitude of the applied stretch as shown above (*cf.* Fig. 10). In relating phase lead to frequency measurements had to be made from recordings with the same amplitude of stretch. In general stretches of medium strength were choosen. However, for frequencies below about 1 c/s the receptor potential evoked by such stretches was generally of too low amplitud to allow exact measurements to be made. The analysis was therefore limited to the frequency range 1—100 c/s. The results are illustrated in the diagram in Fig. 13 *A*. As can be seen the phase lead decreases with increasing frequency and at frequencies above 50 c/s the response is nearly in phase with the stimulus.

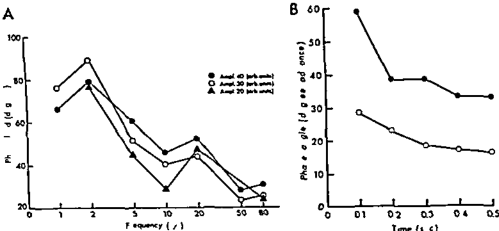


Fig 13 *A* Decrease in phase lead of receptor potential with increasing frequency of stimulation at three amplitudes of stretch *B* Change in phase lead of receptor potential with sinusoidal stimulation at two amplitudes of stretch at 10 c/s

The findings that the phase lead of the receptor potential decreases with increasing frequency while that of the impulse response increases would appear contradictory It has to be noted however that the measurements for the impulse response covered only the range up to 10 c/s The reason why measurements were not made for the impulse response at frequencies above 10 c/s was the fact that the number of impulses at each cycle decreased with increasing frequency and when the stimulus frequency went beyond 10 c/s only 2—3 impulses were produced in each cycle Measurements of peak frequencies and phase lead of the impulse response therefore became impossible for stretches with frequencies above 10 c/s On the other hand within the physiological range of strength and at stimulus frequencies below 1 c/s the receptor potential was usually of too low amplitude to allow exact measurements of its characteristic features to be made By prestretching the spindle so that a steady carrier frequency was produced the number of impulses at higher frequencies of stimulation might have been increased This method however was not used since it would involve the risk of fatiguing the spindle Another reason to avoid prestretch was that earlier studies (Ottoson *et al* 1969) have provided evidence that the response characteristics of the spindle are changed when it is held under constant stretch It is interesting to compare the results obtained on the isolated spindle with those reported by Kirkwood (1972) for the spindle *in situ* although the range for stimulating frequencies is not the same in the two studies Kirkwood found that the phase lead of the impulse response decreased with frequencies higher than about 2 c/s This finding is closely consistent with the observation of a decrease in phase lead of the receptor potential with frequencies above 2 c/s For frequencies below 2 c/s there is an increase in phase lead with increasing frequency for the isolated spindle as well as for the non isolated spindle It would thus appear that with respect to phase lead the isolated spindle behaves closely like the spindle *in situ*

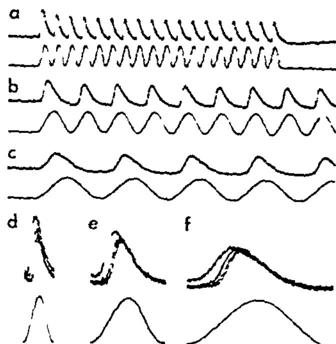



Fig 14 Receptor potentials evoked by trains of sinusoidal stimulation In *d e* and *f* first second fifth and 16th cycle in respective trains are superimposed to illustrate time shift of response

An analysis of the recordings of receptor potentials to trains of sinusoidal stretches reveals several interesting features with important bearings on the mechanisms of change in phase lead of the impulse response This is illustrated in Fig 14 A close inspection of the records *a—c* shows that in the course of sine wave stretching there is a gradual increase in latency of the individual responses This change is accompanied by a parallel shift of the peak of the response towards later phase angle values This can be more clearly seen in *d—f* in which the first the second the fifth and the 12th response in respective trains have been photographically superimposed The change of the responses to the individual sine waves is greatest during the first 1—3 cycles and after 15—15 cycles the response freeze in time and amplitude It may also be noted that while the rise of the individual responses is just shifted to the right the falling phase does not undergo a similar shift

The changes of the receptor potential shown in Fig 14 appear to explain some of the characteristic alteration of the impulse response As described above (*cf* Fig 5 and 6 4) there is an increase in latency of onset of the impulse response to the successive waves of the stimulus train It would appear that this change is accounted for by the delay in onset of the individual receptor potential waves as shown in Fig 14 The characteristic reduction in number of impulses of successive responses in a train may similarly be attributed to the reduction in amplitude of the individual receptor potentials in the train A contributing factor is that the duration of the individual responses shortens as a result of the shift of the rising phase In the course of sine wave stimulation the impulse response suffers a reduction in phase lead (*cf* Fig 4 *B*) It seems most likely that this change of the impulse response is likewise

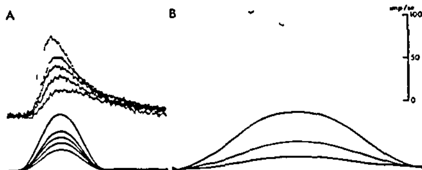



Fig 15 Shift in phase lead of receptor potential (*A*) and impulse response (*B*) with increasing amplitudes of stretch at 50 c/s (*A*) and 0 1 c/s (*B*)

caused by the shift of the receptor potential towards later phase angle values (*cf* Fig 14) In conclusion it would thus appear that the major changes in patterning of the impulse response may be explained by the corresponding changes of the response produced by the transducer elements

c) *Non linear behaviour*

It has been pointed out that the receptor potential to a sine wave stimulus does not reproduce the applied stretch This fact is a strong evidence of the non linear behaviour of the spindle Another evidence for this is the change in phase lead with change in amplitude of stretch As can be seen in Fig 15 *A* there is a shift in phase lead of the receptor potential with increasing amounts of stretch This change appears to account for the changes of the impulse respon e under corresponding conditions As stretch is increased in strength the phase lead of the impulse response increases as shown in Fig 15 *B*

The non linearities of the spindle are also evident when sinusoidal stretches are superimposed upon constant stretches An example of this is shown by the records in Fig 16 *A* A train of sine wave stretch of low amplitude was in this experiment superimposed upon a steplike stretch As seen the sine wave stimulus produced responses which increase with increasing amounts of the underlying maintained stretch Similar effects are produced when a train of sine wave stretching is superimposed upon linearly rising stretch This may be seen in *a* in Fig 16 *B* If the spindle obeyed a linear transfer function the response to the sine wave stretch should have remained unchanged in amplitude However the response to the sine wave stretch became successively higher as the underlying stretch increased The record in *b* gives another example from an experiment in which the sinusoidal stimulus was first applied (for 10 cycles) on a small constant stretch Thereafter the underlying stretch was linearly increased up to a given amplitude and then maintained at this level With the rising stretch the respon e to the sine wave increases in amplitude but then remains constant For a constant amplitude of underlying stretch the respon e to sinusoidal stimulation remains constant during prolonged stimulation as seen in *c*

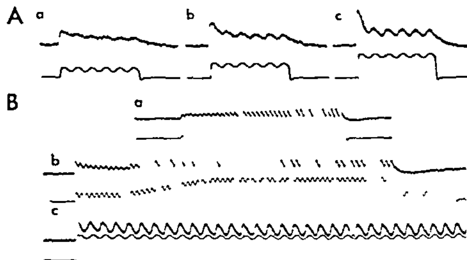



Fig 16 A Increase of receptor potentials to sinusoidal stretch at 50 c/s with increase in amplitude of underlying maintained stretch B Non linear behaviour of spindle Receptor potential to sinusoidal stretch superimposed upon linearly rising stretch (a) In b the underlying stretch was first maintained constant (first 10 cycles) and then increased linearly to maintained level Record c illustrates regularity of response to prolonged stimulation Sine wave stretch superimposed upon maintained stretch Sine wave frequency in a and b 50 c/s in c 70 c/s

## Discussion

Under natural conditions the muscle spindle is most likely subjected to length changes of widely varying time course and amplitude Some of these changes may be of rhythmic nature and may be produced by periodic variations in the fusimotor activity For the understanding of the role played by the muscle spindle in various reflex functions it is therefore important to know its response characteristics during rhythmic lengthening and shortening

An important question in this context is whether or not the isolated spindle behaves in the same way as the spindle *in situ* It may be anticipated that removal of the polar parts of the intrafusal fibres might produce changes of the response characteristics of the spindle The close agreement between the results obtained from the spindle *in situ* (Kirkwood 1972) and those in the present study on the isolated spindle provides strong evidence that this is not so The observations on the isolated spindle may therefore be considered as representative for the non isolated spindle

The study by Kirkwood provides valuable information about the response of the spindle to stimulation frequencies from 0 001 to 100 c/s The present results on the isolated frog spindle extend these data by showing that the characteristic patterning of the impulse response may be attributed to the behaviour of the transducer elements Attention has in the present study been focused on the initial stage of changes during a train of sine waves stretch since it appeared reasonable to assume that these changes reflected more closely what might occur under natural conditions than the steady state generally studied in transfer function analysis The results show that during the first 5– 10 cycles of a sine wave stimulus there occurs a sequence of

regular changes in the impulse pattern involving changes in latency and threshold for the individual spikes The changes of the response are most pronounced for the first 2—5 cycles following this the response changes more gradually and finally the impulse pattern freezes These changes of the response are accompanied by a reduction in number of spikes for the individual cycles This reduction may be quite considerable If the first cycle gives 10 spikes the 10th cycle may only give 3—4 spikes This must impuly that the information conveyed to the central nervous system is much poorer for the later cycles than for the initial ones The reduction in number of impulses for the successive responses in a train can partly be attributed to the changes of the underlying receptor potential and partly to the effect of accumulated refractoriness As shown in Fig 5 the latency for the impulse response is reduced for the first 10—15 cycles and then remains constant The latency of the receptor potential undergoes a similar initial change and then freezes at a given time value This shift of the receptor potential appears also to explain the change in phase lead of the impulse response The close relation between the receptor potential and the various characteristics of the impulse pattern is further evidenced by recordings such as those in Fig 8

A great deal of interest has been focused on the input-output relations of the muscle spindle (*cf* Matthews 1972) and it has been suggested that the spindle obeys a linear transfer function (Brown and Stein 1966 Poppele and Bowman 1970) Analysis of the response of the cat spindle provides evidence however that this may not be so (Lennerstrand and Thoden 1968) or that the linear range is extremely limited (Matthews and Stein 1969) The results of the present study provide evidence that the non linear behaviour can be ascribed to the properties of the transducer elements This is shown by the fact that the form of the receptor potential evoked by a sine wave stretch departs from that of the sine wave stimulus in having a relatively steep rising phase and an exponential fall Another evidence is the finding that the response when superimposed upon a linearly rising stretch (*cf* Fig 15) increases with the underlying stretch The experiments do however not exclude the possibility that the spindle behaves linearly to small amplitudes of sinusoidal stretches If so it seems however that the linear range would be rather limited This conclusion is in agreement with the findings by Matthews and Stein (1969) on the cat spindle

The fact that the receptor potential to sine wave stretch does not have a pure sinusoidal shape may be explained in several ways The most likely explanation would be that the stimulus (applied to the intrafusal muscle fibres) undergoes a distortion during transmission to the endings The departure of the response from the sine wave form would accordingly reflect the influence of the visco elastic properties of the transmitting structures Although mechanical factors most likely contribute to the characteristic configuration of the response it is plausible that other mechanisms are also involved It has been shown earlier (Husmark and Ottoson 1971) that the configuration and time course of the receptor potential is closely related to the ionic events in the transducer process It would be natural to assume therefore that the electrogenic properties of the transducer membrane may influence

the response to sinusoidal stretch This idea lends support from the observations by Pinter (1966) that the receptor potential of the visual cells of the *Limulus* eye to sinusoidal light pulses is distorted as compared with the pure sinusoidal stimulus Like the response of the spindle the receptor potential of the *Limulus* photoreceptor cell has a fast rising phase and a slow falling portion

In conclusion the present study shows that the response of the isolated muscle spindle of the frog to sinusoidal stimulation undergoes characteristic changes in response amplitude latency phase lead and other characteristics during the first 2—5 cycles of stimulation After 10—15 cycles the response pattern freezes The alterations of the impulse response are explained by corresponding changes of the parameters of the underlying receptor potential

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# Rat Liver Glucokinase Activity Studied in Liver Perfusion System

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## Abstract

HODDEVIK G and Ø SPYDEVOLD *Rat liver glucokinase activity studied in liver perfusion system* Acta physiol scand 1974 *90* 41—51

Attempts were made to demonstrate an adaptive increase in glucokinase in isolated perfused livers Several physiological factors such as insulin hydrocortisone high glucose and low fatty acid concentrations which may have a regulatory effect on the adaptive process *in vivo* were tested None of these were sufficient to bring about the adaptation *in vitro* Glucokinase of livers from fasted rats and then perfused with either bicarbonate buffer 100 % blood or with mixtures of these media were studied The concentration of free fatty acids in the media was decreased by addition of insulin and elevated by anti insulin serum. These changes were not accompanied by change in glucokinase activity A moderate decrease in glucokinase in livers from carbohydrate fed rats was observed when the livers were perfused for several hours with subphysiological glucose concentration in the medium The enzyme was quite stable however when glucagon was added to the system The adaptive increase in glucokinase activity could be demonstrated if the fasted rats were refed a carbohydrate diet for 3 hours before the livers were excised However addition of insulin to the perfusion medium was not necessary When the protein synthesis inhibitor cycloheximide was added to the perfusion system the glucokinase from carbohydrate fed liver was quite stable whereas the enzyme in standard diet fed rat livers decreased significantly within 6 hours from the start of the perfusion This indicates a different turnover rate of the enzyme under different physiological conditions

The initial reaction in the metabolism of glucose is the phosphorylation of glucose by adenosine triphosphate (ATP) to glucose 6 phosphate catalyzed by hexokinase The existence of at least four different hexokinases is well established (Gonzales *et al* 1964 Katzen and Schimke 1965 Grossbard and Schimke 1966) designed types I—IV in order of increasing mobility on starch gel electrophoresis type IV corresponds to hepatic glucokinase with a high $K_m$ for glucose The concentration of this hepatic enzyme is low in fated rats but increases to a maximal value in the course of 24—48 hours refeeding of non diabetic animals with a diet rich in carbohydrate and decreases to a minimal value within 3 days starvation or when fed a carbohydrate poor diet These changes in activities are in the following called adaptive changes Evidence for adaptive increase of glucokinase (ATP D glucose phosphotransferase EC 2 7 1 2 ) in rat liver is confined to experiments on whole animals

It is well established that this process is dependent upon insulin as well as glucose (Niemeyer *et al.* 1965).

Additional information of the regulation of glucokinase activity has come from *in vitro* experiments although glucokinase adaptation has not been demonstrated *in vitro*. From liver slice incubation studies it has been concluded that glucose stabilizes the enzyme by decreasing the rate of degradation (Sols *et al.* 1965) and liver perfusion experiments have indicated that insulin can decrease the rate of inactivation (Ruderman *et al.* 1967). The rate of inactivation is increased however when rat liver homogenate is incubated with *octanoate* (Weber *et al.* 1966). This is in line with a regulatory effect of FFA on glucokinase adaptation *in vivo* as the enzyme activity is low in diabetic and fasted animals which have a high FFA level in plasma. This hypothesis is supported by experiments of Ruderman, Lauris and Bolton (1968) who have shown that administration of anti-insulin serum to rats *in vivo* increases plasma FFA and brings about a decrease in glucokinase activity. Simultaneous administration of nicotinic acid prevents the FFA elevation and stabilizes the enzyme.

*The complexity of the adaptation process is emphasized by the regulatory effect* of other hormones. Niemeyer *et al.* (1965) have demonstrated a facilitating effect of hydrocortisone on adaptive increase of glucokinase. Glucagon and adrenalin prevent the process, probably by a repressor effect at transcription level (Niemeyer 1967, Ureta *et al.* 1970). A possible effect on the degradation process is not ruled out.

The present study was undertaken to investigate whether or not any effect of the several suggested regulators of the glucokinase level could be demonstrated *in vitro* with the isolated perfused rat liver.

## Methods and material

*Animals and their treatment.* Male Sprague Dawley rats, raised on a stock diet were used in all experiments. The liver donor rats weighed 110—130 g when the experiments started. The animals were treated as described in the legends to figures and tables. The blood for the perfusion medium was drawn from abdominal aorta of rats weighing 250—300 g. A 20 ml syringe with 100 i.u. heparin was used and 10—12 ml was drawn from each rat. Ether was used for routine anesthesia. Sodium pentobarbital (5 mg per 100 g body weight) was used in some experiments.

*Preparation of homogenates.* Samples from the perfused livers were taken from the distal parts of the lobes. Samples for enzyme determination were homogenized with 10 volumes homogenizing medium in a motor driven Potter Elvehjem homogenizer provided with a loose fitting pestle in ice bath at 0° C. The homogenate was then centrifuged at 10 000 × *g* for 10 min to remove debris. The supernatant was used for hexokinase assay. The homogenizing medium consisted of 0.24 M sucrose, 20 mM EDTA and 3 mM 2-mercaptoethanol at pH 7.5. Liver samples for determination of FFA were immediately homogenized in 2 ml destilled water at room temperature and treated as described by Rose *et al.* (1964).

*Perfusion method.* All perfusions were performed with isolated livers in recyclic systems and with constant pressure (15 cm $H_2O$). Three different perfusion systems were used. The system described by Seglen and Jervell (1969) was used as standard method. 35 ml of the medium was used to establish the cyclic flow. The standard perfusion medium consisted of 1/3 rat blood and 2/3 of an albumin containing buffer composed according to Schimassek (1962). The *glucose concentration* varied from 28 mg to 35 mg per 100 ml. A second perfusion system was used in some experiments in order to perfuse the liver with fresh medium each hour. *The livers* were perfused alternatively in two separate appartus. Whilst the perfusion was conducted in one apparatus the second recyclic system was set up with freshly prepared medium. The liver with the underlying glass disc was then removed to the apparatus with the fresh medium after the vena porta was clamped off to avoid embolism. The liver was out of circulation for about

15 seconds with each changeover The temperature of the medium entering the liver was 37 C in all experiment. The third perfusion system was employed to test the effect of perfusion with 100 % blood To avoid evaporation from the blood which then could be too viscous these perfusions were conducted in a room at 37 C and 95—100 % humidity The livers were arranged in the glass disc above the oxygenator as in the system of Seglen and Jervell (1969) However to minimize the hemolytic changes in the medium that may be due to the mechanic peristaltic pump the blood from the oxygenator was collected in a small glass vessel and poured gently into the upper reservoir containing 150 ml and which was arranged with the meniscus 15 cm above the liver

The viability of the perfused livers was estimated from the appearence the flow the bile production and the utilization of oxygen. The utilization of oxygen by the liver was estimated from the difference in the oxygen content in the input and output medium and flow rate The oxygen content was calculated from the hemoglobin and the $O_2$ pressure determined by a Radiometer $O_2$ electrode The flow rate was measured by counting the frequency of the drops leaving the liver The flow rate was not allowed to be less than 1 ml/1 g/min

*Enzyme assay* The amount of glucose 6-phosphate formed from glucose by hexokinase and glucokinase was determined by following the appearence of NADPH in the presence of purified glucose 6 phosphate dehydrogenase and NADP The details have been described previously (Borrebaek *et al* 1970)

*Chemical analysis* Protein was determined by the method of Lowry *et al* (1951) Glucose was determined with glucose oxidase method (Hugget and Nixon 1957) Free fatty acid (FFA) in perfusate was determined by the method of Dole and Meinertz (1960) FFA in liver tissue was determined in lipid extract after adsorption of phospholipids on silicic acid according to Rose *et al* (1964)

*Materials* Ten times recrystallized glucagon free ox insulin was a gift from Novo Terapeutisk Laboratorium (Copenhagen) Crystalline glucagon was purchased from Sigma Chemical Co (St Louis Mo) The hormone was dissolved in 0 005 N HCl and diluted in bicarbonate buffer containing 1 g albumin per 100 ml to obtain 50 μg glucagon pr ml immediately before use The glucagon solution was infused continuously with a B Braun Melsungen infusion pump at a constant rate of 30 μg per hour (10 μl/min) Anti insulin serum (Guinea pig anti bovine insulin serum) was obtained from Miles Laboratories (Slough England) It was dissolved in distilled water immediately before use Hydrocortisone acetate was from Løvens Kemiske Fabrik (Copenhagen)

## Diets

The standard diet consisted of an adequate stock diet and water The carbohydrate rich diet used in some experiments was a mixture of 40 stock diet 30 glucose and 30 crumbled bread fed *ad libitum* together with 10 % glucose in the drinking water

## Results

*The glucose phosphorylating capacity in different lobes of fed and fasted states* Preliminary experiments indicated different patterns in the distribution of the phosphorylating enzymes in the different lobes of liver in fed and fasted states Therefore some rats fed a carbohydrate rich diet (see MATERIALS) and some starved for 72 h were used to study these distribution patterns Table I shows the enzyme activities in 4 different lobes of the liver In carbohydrate fed state there was no difference in the activities in different lobes However in fasted state somewhat higher activities were observed in the lobes from the left side of the liver This may be due to differences in the distribution of glucose and/or insulin coming from the portal vein Since the enzyme activity in the lobes at the right side show greater differences between the fasted and the fed state liver samples were taken only from the right lobes in the following experiments

*The effect of some physiological factors on glucokinase activity during liver perfu*

TABLE I *The distribution of glucokinase and hexokinase between the different lobes from fed and fasted rats.* 4 rats fasted for 72 h and 4 rats fed a carbohydrate rich diet for 48 h were used. The total phosphorylating capacity in the right posteriorlobe was arbitrarily given the value of 100 % activity in the livers from fed rats and 42 % in those from fasted rats, which is the mean relative to the corresponding value of the fed state. The activities in other lobes were calculated relative to these values in each rat. The relative values were calculated from the specific activities (µmoles NADPH/min per mg protein) and presented together with the S.E. values

| Lobes | Carbohydrate fed rats | | | Fasted rats | | |
|---|---|---|---|---|---|---|
| | Total Phosphorylating activity | Hexokinase | Glucokinase | Total Phosphorylating activity | Hexokinase | Glucokinase |
| Right posterior | 100 | 10 ± 2.6 | 90 ± 1.1 | 42 | 16 ± 1.4 | 26 ± 1.5 |
| Right anterior | 106 ± 3.3 | 11 ± 2.4 | 95 ± 2.3 | 47 ± 1.4 | 15 ± 1.1 | [illegible] ± 0.9 |
| Left lateral | 104 ± 5.4 | 11 ± 2.4 | 97 ± 4.1 | 55 ± 3.7 | 16 ± 1.4 | 33 ± 7.6 |
| Caudate lobe | 103 ± 1.1 | 12 ± 2.2 | 91 ± 1.7 | 56 ± 3.9 | 16 ± 1.2 | 40 ± 5.8 |

sions. No adaptive increase in glucokinase activity could be demonstrated when livers from 3 days starved rats were perfused in the presence of insulin and high glucose concentration, two possible inducers of the enzyme (Fig. 1). It has been suggested that the dietary adaptation may be hampered by adrenalin (Ureta *et al.* 1970) and the absence of hydrocortisone (Niemeyer *et al.* 1965). Mondon and Burton (1971) have shown that lack of cholinergic stimulation reduces the effect of insulin on deposition of glycogen in perfused liver. In order to investigate whether or not any of these factors could cause absence of glucokinase adaptation *in vitro* (Fig. 1) experiments were performed in exactly the same way but the following variation of the experimental procedure were tried: 1. propranolol (an inhibitor of the adrenergic beta receptors) and phentolamin methosulphate (an alphareceptor inhibitor) were administered to the rats 3 h prior to surgery. 2. perfusions were performed with continuous infusion of acetylcholin at a rate of 200 µg/min. 3. one mg of hydrocortisone was added to the perfusion medium every 30 min. None of these alternative experimental procedures caused any significant deviation from the results described in Fig. 1.

Factors necessary for the adaptation process may exist in blood and the concentration of such factors might be too low in the standard medium which contains only 1/3 rat blood (See METHODS). Perfusions were therefore conducted with undiluted freshly drawn blood from carbohydrate fed rats to test this possibility. A special perfusion technique was used in these experiments (See METHODS). Insulin and glucose were added as in the experiments described above. However these experiments did not show any change in glucokinase activity during the experimental period of 8 h. (The results are the same as those in Fig. 1.)

*The effect of the in vivo start of the adaptation process.* The adaptive increase of glucokinase depends upon insulin and glucose and is observed after a lag period

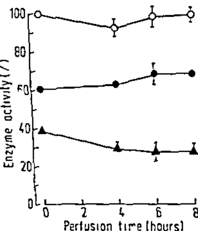



Fig 1 Glucose phosphorylating activity in isolated perfused livers from rats which had been fasted for 3 days The perfusions were performed with a standard medium with addition of glucose to the initial concentration of 300 mg per 100 ml One mU insulin was added every 30 min in 5 perfusions and 100 mU was added every 30 min in 3 perfusions The specific enzyme activities are calculated relative to the initial total activity (100 %) The glucokinase activity (●) is the difference between the total activity (○) and the hexokinase activity (▲) The data represent the means of 8 observations The vertical bars indicate the S E values

(representing the formation of a messenger molecule?) of about 3 h after administration of these components to fasted rats *in vivo* (Sols *et al* 1965) To see if insulin and glucose are necessary for the whole adaptive period or only for the initial process fasted rats were refed a carbohydrate rich diet for 3 h (corresponding to the lag period) and the liver then removed for perfusion

Experiments without any addition of insulin and glucose to the perfusion system showed an increase both in glucokinase and total hexokinase activity (Fig 2) The hexokinase showed an unexpected decrease however No further increase in activities was found when insulin and glucose were added to the perfusion system Thus the

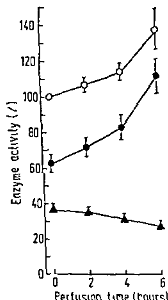



Fig 2 The glucose phosphorylating activity in perfused livers from carbohydrate refed rats The liver donor rats were fasted for 3 days and then refed a carbohydrate rich diet for 3 h before surgery Standard medium was used The perfusions (5) were conducted without addition of glucose or insulin The observations are presented with means and S E values (vertical bars) in the figure The glucokinase activity (●) as estimated as the difference between the total activity (○) and the hexokinase activity (▲)

*in vitro* process leading to increased glucokinase activity does not seem to be dependent upon the presence of these compounds once the process has been started

*The stability of glucokinase in perfusions with bicarbonate buffer* The stability of glucokinase when rat livers were perfused with standard medium containing blood (Fig 1) and the observation of Ruderman *et al* (1967) that the enzyme was unstable when rat livers were perfused for 3 h with a medium not containing blood pointed to a stabilizing effect of blood in the medium To see if this could be confirmed we have run some perfusions (5) for 3 h with bicarbonate buffer with 3 g albumin per 100 ml and no addition of blood However contrary to the results of Ruderman *et al* (1967) the glucokinase was constant in the experimental period (The figures not presented) Thus the stability of the enzyme was not dependent upon blood in the 3 hours perfusion in our hands

*The effect of low glucose in the perfusion medium* A stabilizing effect of glucose may be the reason for the constancy of the enzyme activity in these experiments as glucose concentration increased to high levels (more than 200 mg per 100 ml) within an hour even if no glucose was added due to efflux from the liver When perfusions were performed with replacement of the media each 60 min (See METHODS) the final glucose concentration in the successive media decreased rapidly The final glucose concentrations from two typical experiments are plotted in Fig 3 When glucagon was continually infused at a rate of 30 µg per hour a more rapid glucose release from the livers took place The technique allowed perfusions of livers for some hours with low concentrations of glucose (The glucose concentration in the freshly prepared medium was 30 mg/100 ml) When glucagon was infused the glucokinase activity was unchanged within the experimental period Surprisingly however some

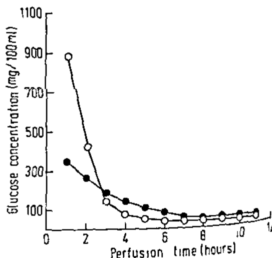



Fig 3 Effect of hourly replacement of perfusion media (See METHODS) on glucose output from carbohydrate fed rat livers during perfusion Standard perfusion media with initial glucose concentration of 30 mg per 100 ml was used The glucose concentration at the end of each hourly perfusion period was measured The figure shows the final glucose concentrations in the media in 2 typical experiments one with continuous infusion of glucagon at a rate of 30 µg/h (O) and one without glucagon (●)

TABLE II *The effect of low glucose containing medium on the hexokinase and glucokinase activity in perfused rat liver* Liver from carbohydrate fed rats weighing 100 g were perfused with low glucosecontaining medium using a technique of medium replacement as described in METHODS The data represent the results of 4 perfusions with glucagon infusion and 4 without The glucagon was infused continuously with 30 μg/h by an infusion pump The activities are calculated relatively to the initial total specific activity (100 ) and are presented together with the S E values

| Time | Glucagon infusion | Total glucose phosphorylating activity | Hexokinase | Glucokinase |
|---|---|---|---|---|
| Initial | – | 100 | 13±0 9 | 87±0 9 |
| 11 h | – | 77±4 5* | 13±1 0 | 64±3 5* |
| Initial | + | 100 | 16±2 3 | 84±2 4 |
| 11 h | + | 100±6 9 | 13±1 7 | 87±7 7 |

* Significant different from the initial value (P < 0 001)
** (P < 0 001)

what lower activity was observed at the end of the 11 h perfusions when no glucagon was added (Table II)

*Perfusions with reduced and elevated FFA concentrations* A regulatory effect of FFA on glucokinase by decreasing the stability has been suggested (Weber *et al* 1966 and Ruderman *et al* 1963) Therefore experiments were carried out to

TABLE III *Effect of anti insulin serum on isolated perfused rat liver* The perfusions were carried out with livers from rats fed standard laboratory diet and rats fed a carbohydrate rich diet for 3 days *ad libitum* Standard perfusion medium was used Samples for determination of enzyme activity and fatty acid were taken immediately after the perfusion started Guinea pig anti bovine insulin serum (2–3 U) was then added to the perfusion system Standard diet fed rats were used for perfusions without AIS addition serving as control of anti insulin serum activity The data (presented together with the S E values) are the results of six perfusions in each group The enzyme activities are expressed as mμmoles NADPH/min per mg protein and the fatty acids as μEq acid/g tissue

| Addition of AIS | Diet of liver donor | Perfusion time | Glucokinase | Hexokinase | FFA in perfusion medium | FFA in liver tissue |
|---|---|---|---|---|---|---|
| – | Standard | 0 | | | 0 42±0 05 | 0 85±9 16 |
| | | 1 h | | | 0 43±0 06 | |
| | | 5 h | | | 0 46±0 06 | 0 82±0 06 |
| + | Standard | 0 | 11 7±0 8 | 2 89±0 67 | 0 43±0 06 | 0 83±0 1 |
| | | 1 h | | | 0 51±0 05 | |
| | | 5 h | 12 3±1 7 | 2 92±0 12 | 0 63±0 06 | 0 81±0 07 |
| + | Carbohydrate | 0 | 23 4±0 96 | 2 95±0 17 | 0 44±0 017 | 0 37±0 02 |
| | | 1 h | | | 0 63±0 014 | |
| | | 5 h | 23 0±1 7 | 3 0 ±0 47 | 0 94±0 16 * | 0 54±0 04* |

Significant different from the initial value (P = 0 05)
(P = 0 01)
(P < 0 001)

TABLE IV *Effect of cycloheximide on glucokinase and hexokinase in perfused rat liver* Livers from 3days carbohydrate fed rats and from rats fed a standard laboratory diet were tested separately The perfusions were performed with standard perfusion medium Samples for determination of the initial enzyme activity were taken immediately after the perfusions were started and 500 μg cycloheximide/30 ml medium was then added The initial total phosphorylating activity in livers from rats fed a carbohydrate rich diet were arbitrarily given the value of 100 activity and in those from rats fed a standard diet 65 which is the mean relative to the corresponding values of carbohydrate fed state The other activities were calculated relative to these values in each rat The data represent the results of 6 perfusions from each dietary group and are presented together with the S F values

| Time | Carbohydrate rich diet | | | Standard laboratory diet | | |
|---|---|---|---|---|---|---|
| | Total phosphorylating activity | Hexokinase | Glucokinase | Total phosphorylating activity | Hexokinase | Glucokinase |
| Initial | 100 | 13 ± 1 1 | 87 ± 1 7 | 65 | 13 ± 1 3 | 52 ± 2 1 |
| 6 h | 101 ± 2 6 | 14 ± 1 5 | 87 ± 2 1 | 49 ± 5 8 | 15 ± 0 9 | 34 ± 6 3* |

* Significant different from the initial value (P = 0 0?)

investigate the relation between the FFA level and glucokinase activity during perfusions When livers were perfused with addition of insulin (1 mU) the FFA level in the medium was reduced to 58 % of the initial value within three hours perfusion There were however small changes in the FFA concentration in the tissue The glucokinase activity was not changed whether insulin was added or not (The figures not given) When livers were perfused with addition of anti insulin serum en increased level of FFA in the medium was observed (Table III) When carbohydrate fed rat livers were used an increase in the tissue concentration was also seen These changes were however not accompanied by changes in hexokinase nor in glucokinase activity

*The effect of protein synthesis inhibitor (cycloheximide)* The stability of glucokinase in the perfusion system when the protein synthesis was inhibited by cycloheximide is shown in Table IV Livers from rats stabilized on standard and high carbohydrate diet respectively were used The table shows that perfusion for six hours with protein synthesis inhibitor brought about reduced enzyme activity in livers from rats fed standard diet whereas the enzyme activity in the rats fed a carbohydrate rich diet for 3 days was not changed This may indicate that there was a higher turnover rate when glucokinase was stabilized at an intermediate level (in the standard diet fed rats) than at maximal activity (in carbohydrate fed rats)

## Discussion

The lack of effect of insulin and glucose on glucokinase adaptation in perfused liver may be due to impaired function of the isolated organ as a result of stress during the operation The adaptation process may involve stimulation at the transcription level

by the inducers (Sols *et al* 1965 Niemeyer 1967) An impaired ability of the perfused liver to respond to inducers at transcription level may therefore be the reason why an adaptive increase could not be demonstrated However the perfused liver is able to respond to corticosteroid at the transcription process (Hager and Kenney 1968) and its seems unlikely that the transcription could be so selectively impaired by manipulation that it respond to corticosteroids and not to insulin

It has been suggested (Pilkis 1970) that insulin acts on the adaptation of glucokinase via an effect on the intermediary metabolism Compounds inhibiting glycogen deposition such as glucagon and adrenalin are inhibitors of glucokinase adaptation (Niemeyer 1967 Ureta *et al* 1970) Isolated perfused liver from fed rats give a very pronounced release of glucose due to glycogen degratation (Burton *et al* 1967 Seglen and Jervell 1969) This suggests an impaired regulation of the intermediary metabolism in the perfused liver in the direction known to inhibit glucokinase adaptation

Fig 3 and Table II indicate some destabilization effect of low glucose concentration When glucagon was added to the system the activity was surprisingly constant even if the livers were perfused for several hours with subphysiological (30—40 mg glucose/100 ml) concentration of glucose A stabilizing effect of glucagon on the enzyme could not be demonstrated however when glucagon was administered to fasting rats *in vivo* (unpublished results)

The presented results point to the possibility that glucokinase activity is only indirectly affected by insulin and glucose But it is also possible that another necessary factor may be lacking in the perfusion system Perfusions with 100 % blood drawn from carbohydrate fed rats and with addition of both insulin and glucose have not shown the adaptation *in vitro* These experiments therefore do not give support to the hypothesis of a stable factor in blood which is sufficient to bring about an adaptation process It is possible however, that necessary labile factors do exist in blood

In an earlier communication from this laboratory (Borrebaek and Spydevold 1970) it was suggested that the level of adipose tissue hexokinase could possibly be regulated by the concentration of FFA A similar suggestion in the case of liver glucokinase has been advanced by Weber *et al* (1966) However the present study does not give evidence in support of this theory Perfusion experiments with anti insulin serum (Table III) show that liver perfusions can be performed with elevated FFA in the medium as well as in the liver tissue without any changes in the glucokinase activity This is not in line with a regulatory effect of FFA on glucokinase but points to differences in the mechanism of adaptation of liver glucokinase and adipose tissue hexokinase

A remarkable fact in the present study is the stability of the enzyme under investigation A tendency of glucokinase to decay has been prominent in *in vitro* experiments performed by others (Sols *et al* 1965 Ruderman *et al* 1967) This may be taken to imply that our perfusions have offered the livers more physiological conditions However we have not been able to start neither an increasing nor a decreasing adaptive process *in vitro* showing that the conditions are still not

sufficiently physiological for a thorough investigation of glucokinase adaptation The difficulty with initiation of both the increasing and decreasing processes may reflect that they are intimately correlated The cycloheximide experiment also imply that the activation and deactivation processes change their rate in a coordinated way It is in this context interesting that Borrebaek *et al* (1964) in x irradiated rats found that activation and inactivation processes involving glucokinase were impaired to about the same degree

A special regulator molecule could be the coordinator of the two processes Further the paradoxical effect of actinomycin on the inactivation rate for glucokinase observed by Sols *et al* (1965) could also be explained if one assume that the formation of such a molecule involves a transcription process The observation (Fig 2) that insulin seem to be necessary only for the initial phase in the adaptive increasing process suggests that a detailed study of this phase may give further information of the effect of insulin on the adaption processes and how they are coordinated

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# Metabolism of $^{14}$C-histamine in Amphibians (Bufo bufo) and Reptiles (Pseudemys scripta and Testudo hermanni)

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## Abstract

ELIASSEN K A and O B REITE *Metabolism of $^{14}$C histamine in amphibians (Bufo bufo) and reptiles (Pseudemys scripta and Testudo hermanni)* Acta physiol scand 1974 *90* 52—56

The metabolism of parenterally administered $^{14}$C histamine was studied in the toad (*B bufo*) the terrapin (*P scripta*) and the tortoise (*T hermanni*) Paper chromatography and isotope dilution techniques were used for estimation of $^{14}$C metabolites in urine Urinary excretion of radioactivity usually exceeded 50 % of the total dose within 24—48 h of administration of $^{14}$C histamine Methylation of histamine to 1 4 methylhistamine and further oxidation to 1 4 methylimidazoleacetic acid was quantitatively the most important pathway for histamine degradation accounting for about 80 and 50 % of the radioactivity in urine of the toad and the reptiles respectively Oxidative deamination of histamine to imidazoleacetic acid also took place in all the studied species In toad urine imidazoleacetic acid was found in the free form only whereas in urine from both reptilian species most of it was bound to ribose

Tissue histamine content and smooth muscle sensitivity to histamine are generally low in amphibians and high in reptiles (Reite 1972) The difference in histamine content which is evident in most tissues except those of the digestive tract is due to the presence of high concentrations of histamine in the mast cells of reptiles whereas amphibian mast cells apparently do not contain this substance (Reite 1969 a Chiu and Lagunoff 1972) The present study was undertaken to elucidate whether the difference between amphibians and reptiles with respect to tissue histamine content and pharmacological effects of histamine is accompanied by any marked difference in histamine metabolism Radioactive metabolites were searched for in urine after parenteral administration of $^{14}$C histamine

## Materials and methods

*Experimental animals* Studies were performed with the European common toad *Bufo bufo* (b w 12—25 g) the Greek tortoise *Testudo hermanni* (b w 300—600 g) and with the American pond terrapin (red eared fresh water turtle) *Pseudemys scripta* (b w 200 —300 g)

The animals were obtained from commercial sources and were kept in the laboratory for 2—3 weeks—at temperatures of 20—22 C with access to water but without food—before being used in experiments

*Administration of $^{14}C$ histamine* The $^{14}C$ histamine (dissolved in physiological saline) was administered intraperitoneally in all animals except two of the tortoises in which the intravenous route was chosen for comparison Tortoises were given 5 μCi in 1 ml saline terrapins 3 μCi in 1 ml saline and toads 0 5—5 μCi in 0 25 ml saline

*Sampling of urine* After administration of $^{14}C$ histamine all tortoises (4) and terrapins (3) were kept in boxes without access to water and none of them urinated during the experimental period (24—48 h) Urine was collected from the urinary bladder by means of a hypodermic needle attached to a syringe after decapitation of the animals and removal of the plastron

In toads three different experimental procedures were followed One series of experiments was performed with the toads kept individually in glass jars without water Single specimens were killed by decapitation at different times (3—48 h) after injection of $^{14}C$-histamine Urination often took place when the toads were decapitated and the discharged urine was collected in the jar together with that still present in the urinary bladder This treatment is later referred o as experimental procedure I In procedure II the toads were treated as described above except that 10 ml of distilled water was put into the jar together with the toad left there during the experimental period (3—144 h) and collected mixed with urine when the toad was killed Procedure III differed from procedure II in that the toads (2 individuals only) were allowed to stay alive for 48 h but they were transferred to new jars with 10 ml of distilled water at intervals of 3—24 h At each such transfer gentle pressure was applied to the abdomen of the toads to achieve emptying of the urinary bladder into the jar from which they were removed All the studied animals were kept at temperatures of 20—22 C throughout the experiments

*Determination of radioactivity of urine* The radioactivity of urine (or mixture of urine and water) was recorded with a liquid scintillation spectrometer after adding 0 1—1 0 ml samples to 10 ml Instagel® scintillation liquid

*Estimation of $^{14}C$ histamine and its metabolites* Two dimentional paper chromatography was used for the estimation of $^{14}C$-histamine and metabolites of $^{14}C$-histamine in urine It proved difficult to separate histamine from 1 4 methylhistamine by means of the paper chromatographical systems In some instances isotope dilution technique was therefore applied to estimate the radioactivity that could be ascribed to each of these two substances The procedures which were followed have been described elsewhere (Eliassen 1969)

*Chemicals* Histamine (2 ring $^{14}C$) spec act 54 mCi/mmol was supplied by Radiochemical Centre Amersham England and Instagel® by Packard Instrument International S A Inc Zurich Switzerland Otherwise the reagents were similar to those used in previous work (Eliassen 1969)

## Results and discussion

*Urinary excretion of radioactivity*

In toads no difference was noticed between results obtained with experimental procedure I and II Some radioactivity was present in urine (or mixture of urine and water) from individuals killed 3 h after administration of $^{14}C$ histamine After 12 h the recovered activity (mean) amounted to 32 % (S D = 11 n = 6) of that administered and the corresponding value after 24 h was 47 % (S D = 18 n = 10) In individuals killed 48 h after administration of $^{14}C$ histamine the recovery usually exceeded 45 % but was always lower than 65 % After that time (72 and 144 h after administration experimental procedure II) there was a decrease in the percentage of recovered radioactivity

Urine collected from toads 3 6 9 12 15 24 and 48 h after $^{14}C$ histamine administration (experimental procedure III) showed a peak in radioactivity in samples obtained 9—24 h after administration More radioactivity seemed to be recovered with experimental procedure III (sampling of urine from the same toads several times) than with experimental procedure I and II (sampling of urine only when the

TABLE I. Urinary excretion of $^{14}C$ activity, unchanged $^{14}C$-histamine and its main metabolites in toads (*Bufo bufo*), tortoises (*Testudo hermanni*) and terrapins (*Pseudemys scripta*) after parenteral administration of $^{14}C$-histamine (Hi, histamine; 1,4-MeHi, 1,4-methylhistamine; ImAA, imidazoleacetic acid; ImAAR, imidazoleacetic acid riboside; 1,4-MeImAA, 1,4-methylimidazoleacetic acid; HiOH, histaminol; AcHi, N-acetylhistamine).*

| | Species and no. of the individual | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *B. b. f.* | | *T. hermanni* | | | *P. scripta* | | |
| | 1 | 2 | 1 | 2 | 3 | 1 | 2 | 3 |
| Length of experimental period (h) | 24 | 144 | 24 | 24 | 48 | 24 | 24 | 48 |
| Route of administration of $^{14}C$-histamine | i.p. | i.p. | i.p. | i.v. | i.v. | i.p. | i.p. | i.p. |
| Percentage of administered dose recovered | 60 | 40 | 67 | 63 | 59 | 22 | 60 | 74 |
| Distribution of radioactivity among metabolites | | | | | | | | |
| Paper chromatographical estimation | | | | | | | | |
| Application point | 3 | 4 | 2 | 2 | 2 | 10 | 10 | 5 |
| Z-spot | 0 | 0 | 6 | 16 | 2 | 12 | 15 | 2 |
| Hi + 1,4-MeHi | 7 | 11 | 10 | 12 | 10 | 6 | 7 | 19 |
| Free ImAA | 1 | 2 | 1 | 1 | 1 | 1 | 0 | 10 |
| ImAAR | 0 | 0 | 34 | 25 | 26 | 16 | 10 | 3 |
| 1,4-MeImAA | 87 | 80 | 47 | 44 | 57 | 57 | 48 | 56 |
| HiOH + AcHi | 1 | 4 | 3 | 2 | 2 | 5 | 7 | 6 |
| Sum | 99 | 101 | 103 | 102 | 100 | 107 | 97 | 101 |
| Isotope dilution assay | | | | | | | | |
| Hi | | 1 | 0.5 | 1 | 0.5 | | | |
| 1,4-MeHi | 10 | 23 | 20 | 37 | 9 | | | 8 |
| 1,4-MeImAA | | | 54 | | | | | |

* Each of the values obtained by paper chromatography is the mean for two chromatograms developed in 2 different two-dimensional paper chromatographical systems and is expressed as per cent of $^{14}C$-activity found on the chromatograms (procedural loss not corrected for). Values obtained by isotope dilution technique (duplicate assays) are expressed as per cent of total urinary activity.

toads were killed). The latter observation—together with the observation that with procedure II there was a decrease in recovery in toads killed more than 48 h after administration of $^{14}C$-histamine—is suggestive that some of the radioactivity of $^{14}C$-histamine may have escaped as $^{14}CO_2$. Evidence that $^{14}C$-histamine or its radioactive metabolites may be transformed to $^{14}CO_2$ has recently been obtained by Fraile, Casas and Alonso (1971) in another amphibian species (*Rana esculenta*).

In one tortoise (no. 4) and one terrapin (no. 1), both killed 24 h after administration of $^{14}C$-histamine, rather small amounts (14 % and 22 %, respectively) of the administered radioactivity were recovered with the urine. The saline containing the $^{14}C$-histamine was to be given intraperitoneally, but in these two cases it may actually have been deposited subcutaneously due to our being overly cautious to avoid injecting it into the urinary bladder or the lung. All other tortoises and terrapins excreted at least 59 % of the administered radioactivity during the first 24—48 h after administration of $^{14}C$-histamine, as shown in Table I (tortoise no. 4 not listed because its urine was not examined for metabolites).

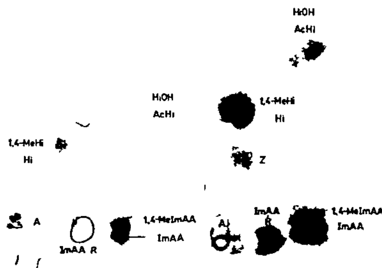



Fig 1 Rad oautograms of two-dimensional chromatograms of the urine from toad (left) and tortoise (right) after parenteral administration of $^{14}$C-histamine The solvents u ed were n butanol acetic acid water (4 1 1 v/v/v) in the first direction (→) and n butanol saturated with 20 % NH3 in the second direction (↑) "A is the application point

*Presence of $^{14}$C histamine and its metabolites in urine* The estimation of $^{14}$C histamine and its metabolites by means of paper chromatography was less adequate for the urine of toads tortoises and terrapins than it has been shown to be for goat sheep and cow urine (*cf* Eliasen 1969 1971) However the method seemed useful for a rough quantitation

Most urine samples examined for metabolites contained more than 50 % of the administered radioactivity A considerable variation in the percentage of the different metabolites was found but methylation and further oxidation to 1 4 methylimidazole acetic acid (1 4 MeImAA) seemed to be the main metabolic pathway in tortoises and terrapins as well as in toads (Table I) Imidazoleacetic acid (ImAA) mostly in the form of riboside (ImAA R) also accounted for a substantial part of the radioactivity of urine in tortoises and terrapins In toad urine ImAA was quantitatively less important and was found only in the free form (see Fig 1) The isotope dilution assay indicated that a very small part of the administered $^{14}$C histmine was excreted unchanged

Two spots on the X ray film had $R_f$ values different from those of the known histamine metabolites One of these spots Z (Fig 1 right) accounted for up to 16 % of the radioactivity on the chromatograms Results obtained with the isotope dilution technique showed that the Z spot could be ascribed to 1 4 methylhistamine (1 4 MeHi) An unusual $R_f$ for part of the 1 4 MeHi may have been caused by interfering substances in the urine

According to the present results the toad the tortoise and the terrapin are all capable of a fairly rapid degradation and excretion of radioactive metabolites of parenterally administered $^{14}$C histamine The metabolic pathways for $^{14}$C histamine also seem to be qualitatively similar in the three species Mammals have previously been shown to inactivate histamine along the same pathways (Schayer 1959) It thus appears that conspicuous changes in smooth muscle responses to histamine—from negligible effects (toads cf Reite 1969 b) to strong inhibitory and excitatory effects (tortoises and terrapins cf Reite 1970)—may have occurred without marked changes in the metabolism of histamine

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# The Distensibility of the Resistance Vessels in Spontaneously Hypertensive Rats (SHR) as Compared with Normotensive Control Rats (NCR)

By

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## Abstract

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Earlier studies on human essential hypertension as well as on SHR suggest that an increased media thickness partly encroaching upon the vascular lumen forms the main background of the increased flow resistance in primary hypertension (*e.g.* Folkow *et al.* 1970). In the present investigation a comparison was made concerning the distensibility of the resistance vessels in order to further explore the proposed difference in vascular design. The simultaneously perfused hindquarter vessels of SHR and NCR were exposed to sudden, repeated shifts of pressure and flow, first at maximal vasodilatation and then at stable levels of smooth muscle tone induced by barium ions or noradrenaline. Relative changes of vascular internal radius ($r_i$) were calculated from the pressure-flow values, assuming $r_i$ to be proportional to $\sqrt{\text{flow conductance}}$. For given changes in distending pressure ($P_D$) the SHR resistance vessels exhibited considerably smaller percentual $r_i$ changes than the NCR ones, whether starting from the same $P_D$, the same $r_i$ or even when both $P_D$ and $r_i$ were initially kept identical in NCR and SHR by exposing the SHR vessels to lower concentrations of constrictor agents. This evidence of reduced vascular distensibility in SHR was obvious both at maximal dilation and particularly when smooth muscle tone was increased. This strongly supports the view that especially the media component of the SHR resistance vessels is enhanced in bulk, thus markedly influencing both active and passive resistance changes.


The wellknown increase of vascular resistance in established primary hypertension both in man and in the spontaneously hypertensive rat, SHR (Okamoto 1969), appears to be mainly due to a structural change of the systemic resistance vessels. Like the obvious hypertrophy of the left ventricle this vascular change seems to represent a *per se* normal and fairly rapid tissue adaptation to increases in functional load (*e.g.* Folkow *et al.* 1972, 1973). It results in an increased wall/lumen ratio, apparently to a great deal due to media hypertrophy, at least initially, and so organized that it in most circuits partly encroaches upon the lumen even at maximal dilatation. It seems likely that such a change should affect vascular distensibility as well, in a way which may have considerable hemodynamic consequences.

Earlier studies of vascular distensibility in hypertension have, however, dealt with mainly larger systemic arteries. Thus Feigl, Peterson and Jones (1963) found a reduced distensibility of the femoral artery in dogs made hypertensive by renal artery clipping as compared to the prehypertensive state. Greene et al. (1966) have reported a reduced distensibility of e.g. the brachial artery in patients with essential hypertension compared with those of normotensive controls. Further, Peterson (1966) and particularly Aars (1968) have presented strong evidence in favour of the view that the important baroreceptor "resetting" in hypertension is essentially a consequence of a rapid structural change of the corresponding arterial walls, reducing their distensibility.

In the present investigation the distensibility of the systemic resistance vessels *proper* has been studied in spontaneously hypertensive rats (SHR) and normotensive control rats (NCR). The technique used was essentially that of Folkow and Löfving (1956) who studied the distensibility of the resistance vessels in cats by perfusing hindlimb preparations over a range of distending pressures and at different levels of vascular tone. In order to provide further information concerning the altered behaviour of the resistance vessels in primary hypertension, the SHR resistance vessels were compared with those of NCR, first at maximal vasodilatation and then at increasing stable levels of vascular smooth muscle activity.

## Methods

In 22 paired experiments the isolated hindquarter vascular beds of one SHR and one matched NCR (6–9 months of age) were simultaneously perfused. Before this preparation the "resting" blood pressure was determined under Nembutal anesthesia (5 mg/100 g b.w.) in the caudal artery via a cannula connected to a Statham pressure transducer and a Grass recorder, thus checking previously performed indirect pressure measurement.

Since the paired hindquarter preparation has been described in detail elsewhere (Folkow et al. 1970), it will only be summarized here. After complete isolation from the upper part of the body by mass ligatures, the two hindquarters were perfused via the abdominal aorta from a double Harvard constant flow pump using oxygenated Tyrode solution at 38° C with addition of 4 % Ficoll (a synthetic colloid polymer of sucrose and epichlorohydrin, m.w. 47 000, AB Pharmacia, Uppsala, Sweden) to minimize oedema formation.

While perfusion pressure was continuously recorded from the tail artery, flow was suddenly shifted in a random fashion to higher and lower values around the "basal" flow level of about 10 ml/min/100 g. Each series of "flow run" comprised 4–8 different flow levels and each of these were checked 2–3 times. Such a flow run was first performed while the vascular smooth muscles were kept completely relaxed by means of large doses of papaverine, i.e. at maximal vasodilatation.

In those animals where e.g. oedema formation had not interfered significantly with the preparation, stable known levels of smooth muscle activity were created, sometimes in the form of one "low" and one "high" level of tone, roughly 3 times and 5 times respective resistances at maximal dilatation for the two groups of rats when measured at the "basal" flow level of 10 ml/min/100 g. The graded vasoconstrictions were achieved by adding suitable concentrations of $BaCl_2$ or adrenaline to the perfusate. When flow resistance had stabilized at the wanted level the series of sudden flow changes was repeated.

The virtually immediate vascular distensions induced when pressure was suddenly raised were sometimes followed by far slower and smaller "yield" in resistance, which were reproduced as transient, small reductions in pressure "baseline" once flow was suddenly lowered again. To make the influence of these secondary slow and small shifts of resistance (which in relative terms were identical in SHR and NCR) as small and constant as possible, the points in the pressure-flow recordings chosen for deductions of flow conductance were taken in a standardized fashion just after the rapid phase of resistance change had been completed. Precautions were also taken not to go up to such high flow levels that the maximal contractile

strength of the resistance vessels (see Folkow *et al* 1970) was surpassed First towards the end of the measurements flow was increased beyond the level where the resulting pressure load exceeded the maximal contractile strength of the vessels This led to a rapid marked yield in vascular tone obvious from the abrupt bends in the pressure flow curves usually occurring at 200—230 mm Hg in NCR and at 300—350 mm Hg in SHR (see below)

Sometimes the addition of constrictor agent was so adjusted as to result in virtually identical resistance values at the same initial distending pressure in SHR and NCR which called for substantially *lower* concentrations of the constrictor agent to SHR thanks to their hyper reactive resistance vessels (*cf* Folkow *et al* 1970) Then vascular distensibility could be deter mined also in this situation where both average vascular internal radius and distending pres sure should be the same in SHR and NCR

*Calculations of vascular distensibility* From the plotted pressure flow curves of each pair of hindquarter vascular beds relative changes of average internal radius ($r_i$) of the resistance vessels were calculated on the assumption that $r_i$ is proportional to the fourth root of flow conductance according to Poiseuille s Law In this calculation it is assumed that the viscosity of the Newtonian perfusate remains constant and that inertia losses are negligible (*cf* Benis Usami and Chien 1970 Eliassen Folkow and Öberg 1973) over this range of the pressure flow curve The changes in $r_i$ were then plotted against mean vascular distending pressure ($P_D$ equal $\frac{P_A + P_V}{2}$ $P_V$ being in this preparation virtually zero) for each paired experiment after which vascular distensibility *i e the percentual r changes for a cha ge in $P_D$ of 10 mm Hg* was calculated Finally the differences in vascular distensibility at the various levels of tone employed for the paired vascular beds were statistically analyzed by the method of pairing design test

## Results and Comments

Mean arterial pressure measured in the caudal artery of the anesthetized animals was 195±5 mm Hg in the 22 SHR and 125±3 mm Hg in the matched 22 NCR a difference of 57 % These values corresponded well with indirect measurements of

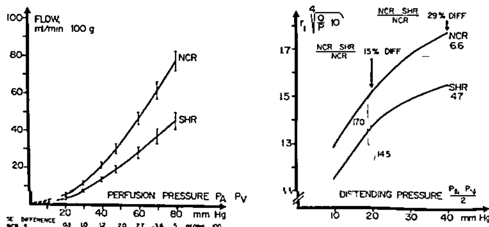



Fig 1 Left part illustrates the mean pressure flow relationship of the maximally dilated hind quarter vascular beds of 8 paired expts (see text) on SHR and NCR The bars on the curves indicate S E M with in the respective group S E M within the paired expts most relevant for the present problem is given below the abscissa in ml/min×100 g In the right part the pressure flow curve for NCR and SHR are recalculated to illustrate the relationship between distending pressure ($P_D$) and the average internal radius ($r_i$) of the respective sets of resistance vessels From these $P_D$ r curves the difference in distensibility of the maximally dilated SHR NCR resistance vessels is illustrated Note that the difference in distensibility between the two curves in r with increasing distending pressures For details see text

tal blood pressure by means of a colorimetric method especially concerning the resistance pressure difference between SHR and NCR, which is here of particular relevance.

In all 22 expts. pressure flow determinations were first made during maximal vasodilatation. It was possible to obtain particularly many points of measurements in 8 of the paired expts. making these pressure flow curves especially reliable as to their configuration. The results of these 8 expts., which well correlate with those of the other expts., are shown in Fig. 1 as the mean pressure flow curves (left part) for the SHR NCR vascular beds during complete smooth muscle relaxation. In the right part of Fig. 1 the pressure flow curves are recalculated to illustrate the relationship between distending pressure ($P_t$) and an expression ($\sqrt[4]{\text{conductance}}$) for the average internal radius ($r_i$) of the resistance vessels. It is seen that vascular distensibility is lower in SHR than in NCR during maximal dilatation and that this difference in distensibility tends to increase when $P_D$ is higher. [illegible] difference in the figure. It is here likely that the r changes at the higher $P_t$ levels, i.e. beyond the tendency of a "bend" on the r curves at 15–20 mm Hg, better represents the true wall distensibility of the relaxed resistance vessels. At lower $P_t$ levels an element of luminal collapse may contribute, just like the more thinwalled veins exhibit partial collapse below transmural pressures of 6–8 mm Hg (e.g. Oberg 1967). In any case, the SHR resistance vessels are obviously stiffer, stiffness being the inverted value of distensibility, than the NCR ones, also at complete smooth muscle relaxation when non-muscular wall elements are likely to offer the main resistance to the distending force.—The average results for the total maximal dilatation calculated for a $P_t$ increase from 10 to 20 mm Hg also exhibits a significant difference ($p < 0.01$, Table I) between SHR and NCR.

TABLE I. Illustrates the mean values with ± S.E.M. of arterial blood pressure and distensibility of the resistance vessels for the SHR and NCR groups together with the significance of the difference between them. The mean of the difference in distensibility between SHR and NCR in each paired experiment is also given as % change in SHR, i.e. $\frac{SHR - NCR}{NCR} \cdot 100$

| | Arterial blood pressure mm Hg | Distensibility of resistance vessels ($\Delta r$ / $\Delta P_D$) During max. vasodilatation | During vascular smooth muscle tone | | |
|---|---|---|---|---|---|
| | | $P_D$ 10–20 mm Hg | Equal $P_D$ 45–100 mm Hg | Equal r $\Delta P_D$ = 10 mm Hg | Equal $r_i$ and $P_D$ $\Delta P_D$ = 10 mm Hg |
| SHR | 1[illegible] | 12.0 ± 0.8 | 3.5 ± 0.4 | 3.9 ± 0.3 | 4.8 ± 1.0 |
| NCR | 125 ± 4 | 14.[illegible] ± 1.1 | 5.5 ± 0.[illegible] | [illegible].2 ± 0.5 | 9.1 ± 1.1 |
| Sign. | p < 0.001 | p < 0.01 | p < 0.005 | p < 0.001 | p < 0.005 |
| % change in SHR | [illegible] ± [illegible] | −11 ± [illegible] | −3[illegible] ± [illegible] | −45 ± 3 | −4[illegible] ± 6 |

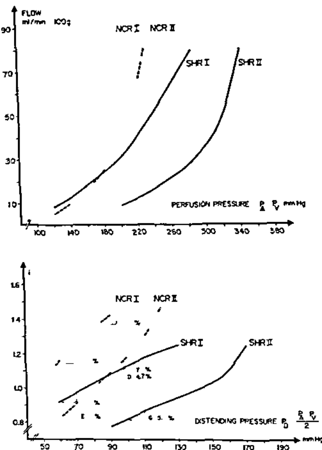



Fig 2 Upper part shows the mean pressure flow curves of 3 representative experiments during 2 levels of vascular tone in NCR and SHR arterial blood pressure being in average 45 % higher in SHR. Curves labelled NCR I and SHR I represent a fairly low level of flow resistance about 3 times that at maximal dilatation when measured at a flow of 10 ml/min × 100 g. In curves NCR II and SHR II the resistance is increased about 5 times that at maximal dilatation. Lower part illustrates the relationship between distending pressure ($P_D$) and average internal radius ($\bar{r}$) as calculated from the pressure flow curves in the upper part. Comparisons are made between NCR and SHR concerning vascular distensibility ($\Delta\bar{r}$ c/$\Delta P_D$) at the same initial $P_D$ (A and C) at the same initial $r_i$ (E and G) and when both $P_D$ and $r_i$ are initially the same (D and F). For further information see text.

The difference in vascular distensibility was more pronounced when the smooth muscles of the resistance vessels were in a state of contraction ("vascular tone"). Particularly when this tone is pronounced the coupled actomyosin complexes would tend to take over the resistance to distension by unloading other non muscular wall elements, in fact even the innermost muscle elements to some extent (Van Citters, Wagner and Rushmer 1962).—The upper part of Fig 2 illustrates at two different levels of stable vascular tone in SHR and NCR the average pressure flow curves for 3 expts in which closely similar levels of vascular tone had been achieved. The mean arterial pressure for these particular animals was 130 mm Hg for NCR and 187 mm Hg for SHR, a difference of 45 %. Subsequent to measurements during maximal dilatation (not shown) vascular tone was first raised to a level where flow conductance was reduced to roughly 30 % of that at maximal dilatation when measured at 10 ml/min × 100 g, here called "low" vascular tone. A complete set of pressure flow run was performed at this level of tone (NCR I and SHR I in Fig 2). By increasing the concentration of the vasoconstrictor agent in the perfusate vascu

tail blood pressure by means of a colorimetric method especially concerning the relative pressure difference between SHR and NCR which is here of particular relevance

In all 22 expts pressure flow determinations were first made during maximal vasodilatation It was possible to obtain particularly many points of measurements in 8 of the paired expts making these pressure flow curves especially reliable as to their configuration The results of these 8 expts, which well correlate with those of the other expts are shown in Fig 1 as the mean pressure flow curves (left part) for the SHR NCR vascular beds during complete smooth muscle relaxation In the right part of Fig 1 the pressure flow curves are recalculated to illustrate the relationship between distending pressure ($P_D$) and an expression ($\sqrt[4]{\text{conductance}}$) for the average internal radius ($r_i$) of the resistance vessels It is seen that vascular distensibility is lower in SHR than in NCR during maximal dilatation and that this difference in *distensibility tends to increase when* $P_D$ *is higher (15 versus 29 %* difference in the figure) It is here likely that the r changes at the higher $P_D$ levels (i e beyond the tendency of a bend on the r curves at 15—20 mm Hg) better represents the true wall distensibility of the relaxed resistance vessels At lower $P_D$ levels an *element of luminal collaps may contribute* just like the more thinwalled veins exhibit partial collapse below transmular pressures of 6—8 mm Hg (e g Öberg 1967) In any case the SHR resistance vessels are obviously stiffer (stiffness being the inversed value of distensibility) than the NCR ones also at complete smooth muscle relaxation when *non muscular* wall elements are likely to offer the main resistance to the distending force —*The average results for the total material* at maximal dilatation calculated for a $P_D$ increase from 10 to 20 mm Hg also exhibits a significant difference ($p < 0 01$ Table I) between SHR and NCR

TABLE I *Illustrates the mean values with* ± S E M *of arterial bloodpressure and* distensibility of the resistance vessels for the SHR and NCR groups together with the significance of the difference between them The mean of the difference in distensibility between SHR and NCR *in each paired experiment is also given as % change in SHR i e* $\frac{\text{SHR}-\text{NCR}}{\text{NCR}} \times 100$

| | Arterial *blood* pressure mm Hg | Distensibility of resistance vessels $\Delta r_i / \Delta P_D$ | | | |
|---|---|---|---|---|---|
| | | During max vasodilatation | During vascular smooth muscle tone | | |
| | | $P_D$ 10−20 mm Hg | Equal $P_D$ 90−100 mm Hg | Equal $r_i$ $\Delta P_D = 10$ mm Hg | Equal $r_i$ and $P_D$ $\Delta P_D = 10$ mm Hg |
| SHR | 195 ± 5 | 12 0 ± 0 8 | 3 5 ± 0 4 | 3 9 ± 0 3 | 4 8 ± 1 0 |
| NCR | 125 ± 3 | 14 7 ± 1 1 | 5 5 ± 0 7 | 7 2 ± 0 5 | 9 1 ± 1 1 |
| Sign | $p < 0 001$ | $p < 0 01$ | $p < 0 005$ | $p < 0 001$ | $p < 0 005$ |
| % change in SHR | +57 ± 5 | −11 ± 7 | −33 ± 6 | −45 ± 3 | −48 ± 6 |

extreme pressure load (*cf* Folkow *et al* 1970) just like every muscle can reach a point of load where its strength is surpassed This yield of the contractile state to an overwhelming load was also clear from the fact that a sudden return to a sub maximal $P_D$ level revealed a transient $r_i$ increase far beyond the $r_i$ value normally present at this particular $P_D$ This yield phenomenon is therefore entirely different in nature from a mere passive stretch of the media when it is in a state of steady contraction as occurs when the $P_D$ rises are not excessive

The lower part of Fig 2 also illustrates how the relationship between $P_D$ and $r_i$ is affected when the same vascular bed displays different levels of tone and hence functionally induced differences in $w/r_i$ Segments A and E on NCR curves I and II respectively can here be compared where in both cases $P_D$ is raised from 60 to 70 mm Hg The percentage $r_i$ increase is somewhat larger when vascular tone is higher ($\Delta r_i$ being 11 % *vs* 9 %) It seems however unlikely that the individual smooth muscle should become *more* distensible along with an accentuated contrac tion since direct measurements on individual small arteries (Wiederhielm 1965) suggest that wall stiffness is increased along with an accentuated smooth muscle activation It may rather be a result of a certain unloading of inner wall layers by the outer ones when the media activety is enhanced (*cf* van Citters *et al* 1962) as combined with the functional $w/r_i$ increase *per se* as mentioned earlier Particu larly this latter view is supported by the fact that calculated changes in either r or in $\frac{r_e+r_i}{2}$ performed as outlined above become *reduced* when vascular smooth muscle tone is raised This strongly suggests that media stiffness in reality *increase* the higher the vascular tone

When segment A of NCR I is instead compared with segment C of SHR I at the same $P_D$ rise as above the r increase is here about 30 % smaller in SHR ($\Delta r_i$ being 6 % for SHR *vs* 9 % for NCR) The combined results as given in Table I are closely similar thus suggesting stiffer walls in the SHR resistance vessels when they are exposed to an equal $P_D$ as the NCR ones and to equal concentrations of con strictor agents

The distensibility of the NCR SHR resistance vessels can be compared at similar concentrations of constrictor agent also by setting out from the same initial $r_i$ (*e g* segments E and G) but the initial $P_D$ levels are then as different as 60 and 100 mm Hg respectively However since these $P_D$ levels correspond closely to the NCR SHR arterial pressures present *in vivo* (see above) such a mode of comparing vascular distensibility is of considerable interest For an equal $P_D$ increase in segments E and G it is shown that the SHR resistance vessels are in this situation about twice as stiff as the NCR ones ($\Delta r$ being 11 % for NCR *vs* 5 4 % for SHR at a $\Delta P_D$ of 10 mm Hg) Again these results are of equal magnitude as the mean values for the entire material (Table I)

Still another way of comparing the distensibility of the NCR SHR resistance vessels in Fig 2 is to start from a situation where both r and $P_D$ are initially the same for the two vascular beds which as mentioned above calls for less constrictor agent

to the SHR resistance vessels. This is perhaps the most interesting approach of all since most current theories assume that the essential difference between normotensive and hypertensive resistance vessels should be a matter of different smooth muscle activities. If such theories were correct the NCR SHR vessels would be closely similar both in activity level, in design and hence in distensibility, at the point where the r curves of NCR II and SHR I cross each other (see lower part of Fig. 2). However, also in this situation the SHR vessels are about 65 % stiffer than the NCR ones $\left(\left(\frac{7.7}{4.7}-1\right) 100\right)$ particularly when the mean difference in stiffness is related to the mean difference in arterial pressure between NCR and SHR (Table I). Thus a comparison of segments D and F reveals that a 10 mm Hg $P_{t}$ increase results in an increase of 7.7 % in NCR but in only 4.7 % in SHR. The figure also illustrates that curve NCR II begins to yield at a $P_{t}$ of about 100 while curve SHR I exhibits no tendency to bend in this $P_{t}$ range, illustrating that the SHR vessels can withstand decidedly higher $P_{t}$ levels without yielding. Actually the two mentioned characteristics in the configurations of curves NCR II—SHR I strongly point to a considerable difference between the NCR—SHR resistance vessels with respect to structural design. The media of the SHR ones is evidently both stronger and stiffer than in NCR and nearly in proportion to the respective difference in arterial pressure between SHR and NCR.

To summarize the above considerations it follows from the data of Fig. 2, lower part, that the SHR *resistance vessels are considerably stiffer (and stronger) than the* NCR ones, independently of which of three different alternatives for relating $\Delta r$ to $\Delta P_{t}$ that one prefers for judging their distensibility. When mean values for the entire material of measurements are calculated (Table I) the following figures for difference in vascular distensibility are obtained in the current groups of NCR and SHR which, as a total material, differ by 57 % in arterial pressure. At identical concentrations of constrictor agents the SHR resistance vessels are $33 \pm 6$ % less distensible than the NCR ones (or, when instead expressed in terms of stiffness $\frac{1}{\text{distensibility}}$, about 50 % stiffer than the NCR resistance vessels), when setting out from the same initial $P_{t}$. The difference in distensibility is $45 \pm 3$ % if one instead starts from the same initial r (the SHR resistance vessels then being 80 % stiffer than the NCR ones). When both r and $P_{t}$ are initially the same for NCR—SHR (but concentrations of constrictor agent differ) the SHR vessels are $48 \pm 6$ % less distensible *(or 90 % stiffer) than the NCR ones.*

## Discussion

The present *findings concerning a reduced* distensibility of the resistance vessels in spontaneously hypertensive rats (SHR) are in good agreement with findings on larger arteries in other types of hypertension. For example, studies *in vivo* on the dog

femoral artery before and during renal hypertension (Feigl, Peterson and Jones 1963) as well as on the exposed brachial artery of man with essential hypertension (Greene *et al.* 1966) indicate a reduced wall distensibility in these conduit arteries. Under such *in vivo* circumstances it may, however, be difficult to settle whether the reduced distensibility reflects mainly an increased smooth muscle tone or predominantly a structural vascular change. On the other hand, large arteries have been studied also *in vitro* when it is easier to control the state of smooth muscle activity, and such studies, too, indicate a reduced wall distensibility in hypertension (Asteroth and Kreuziger 1951, Karnbaum 1961, Vars 1968).

Folkow and Löfving (1956) studied the distensibility of the resistance vessels proper in normotensive cats by perfusing the isolated calf with a plasma substitute and measuring the pressure-flow relationship at various levels of smooth muscle activity, an approach largely adapted in the present study. The mean distending pressure ($P_D$) was here defined as the sum of the arterial inflow and venous outflow pressures divided by two $\left(\frac{P_A + P_V}{2}\right)$ but, since $P_V$ was kept virtually at zero to minimize edema formation, $P_A$ was the essential determinant of intravascular pressure changes. Studied in this way, the distensibility of the resistance vessels was first expressed as percentual changes in total regional flow conductance per unit $P_D$ change, thus providing a resultant of the distensibility of all the consecutive resistance sections, where the precapillary one for geometrical reasons is known to dominate. After this the average internal radius ($r_i$) was calculated from the Poiseuille law relating percentual $r_i$ changes to unit $P_D$ changes.

Theoretically, also changes in vascular length will affect the calculated values for radial distensibility, though concomitant length changes would tend to underestimate the true $r_i$ changes, if anything. In any case, such a source of error is probably minor since the main resistance sections of most vascular beds appear to be "prestretched" by their very arrangement in the tissue, implying minimal shifts in length along with $P_D$ shifts. Only for the highly unlikely case that the SHR resistance vessels would display a markedly increased longitudinal distensibility compared with the NCR ones, the present calculations of "passive" $r_i$ changes would contain a substantial error.—As mentioned earlier, errors due to fluid viscosity and inertia factors can be largely ruled out.

It is, however, also possible that locally induced changes in vascular smooth muscle tone might have interfered, since pressure alterations in blood perfused vascular beds *in vivo* usually provoke such autoregulatory shifts in vascular tone. If so, they would proportionally mask or completely overrule the physical distensibility of the resistance vessels. On the other hand, such "active" adjustments are easily revealed by the characteristic pattern of flow changes occurring when rapid shifts in pressure are induced (*cf.* Folkow and Löfving 1956). Furthermore, they are usually entirely abolished when artificial plasma substitutes are used with addition of either papaverine or stable concentrations of constrictor agents like barium ions. In the present experiments there were never any signs of autoregulatory compensations for the

physical distensibility of the resistance vessels. Thus also such a potential source of error could be safely ruled out, implying that the calculated percentual $r_i$ changes per unit $P_D$ changes reflect in a reasonably correct way the physical distensibility of the NCR—SHR resistance vessels.

Measurements were first performed when the vascular smooth muscles were completely relaxed, in which state they seem to be almost "plastic", so that the main resistance to stretch would then be offered by the elastic and collagen wall elements (*cf.* Folkow and Löfving 1956, Wiederhielm 1965). In agreement with previous results (Folkow *et al.* 1970), the SHR vascular bed offered an increased resistance to flow compared with the NCR one also in this state. Moreover, the percentual $r_i$ increase per unit $P_D$ increase was clearly smaller in SHR than in NCR, implying a truly enhanced stiffness of the SHR resistance vessels even when the media was completely relaxed. It has the additional consequence that the difference in resistance at maximal dilatation between NCR—SHR vascular beds tends to become more marked when compared at higher distending pressures (see Fig. 1).

The stretch exerted by $P_t$ on the different wall elements in resistance vessels is likely to change markedly along with a media contraction. The reason is that the coupled actomyosin complexes will then largely take over, increasing w/r and hence vascular geometry and further unloading more or less other wall elements. In this situation the difference in vascular distensibility between NCR and SHR becomes more pronounced. It can be deducted from *e.g.* Fig. 2, upper part, that the tone levels elicited allowed a flow of plasma substitute of about 10—30 ml/100 g×min at pressures corresponding to the respective arterial pressures *in vivo*. Since the viscosity of the present perfusate was roughly 40 % of that of blood *in vivo* (*cf.* Baeckström *et al.* 1971), the corresponding blood flow levels would be 4—13 ml/100 g×min in the hindquarters (mainly skeletal muscle), *i.e.* it would roughly correspond to the range of vascular tone met with during resting steady state in such a tissue. Therefore, the state of smooth muscle contraction artificially induced for the measurement of vascular distensibility was reasonably representative for the resting situation *in vivo*.

The distensibility of the NCR—SHR resistance vessels was then compared in three principally different ways while in the mentioned state of steady contraction. In the first case, the comparison was made from the same initial $P_D$ level and when both vascular beds were exposed to identical concentrations of constrictor agents. Since there is no difference in vascular "threshold sensitivity" between NCR—SHR, it is likely that largely corresponding level of smooth muscle activity were at hand (*cf.* Folkow *et al.* 1970). Nevertheless, as judged by the *r* changes, the SHR resistance vessels were then about 50 % stiffer than the NCR ones, as related to a 57 % difference in "resting" arterial pressure.

Also in the second case the same concentrations of constrictor agent were used, but the comparison of vascular distensibility was now made from the same initial *r*. Then the respective $P_D$ levels closely approximated those to which the two vascular beds were exposed to *in vivo*, to judge from their "resting" arterial pressures. From

one point of view this approach may imply the physiologically most relevant way of comparing vascular distensibility in normotensive and hypertensive states When compared in such a way the SHR resistance vessels were about 80 % stiffer than the NCR ones

In the third case vascular distensibility was compared from the same initial $P_D$ and $r_i$ in SHR and NCR which necessitated that the SHR vessels were exposed to proportionally lower concentrations of constrictor agent This approach is particularly interesting and revealing because most current theories concerning the background of the raised flow resistance in hypertension ascribe this resistance increase mainly or entirely to an accentuated vascular smooth muscle activity If this were strictly correct the SHR—NCR resistance vessels would be in an identical state concerning function structure and geometry when both $P_D$ and $r_i$ were initially the same They would then also be expected to display largely the same distensibility However even in this situation the SHR resistance vessels were some 90 % stiffer than the NCR ones (see also Table I)

Thus however measured the distensibility of the SHR resistance vessels was considerably reduced when compared with the NCR ones This was especially the case when smooth muscle activity was present adjusted to levels which largely corresponded to those present in the resting conditions *in vivo*, but also in the state of complete smooth muscle relaxation Particularly in the situation where the essential wall resistance to stretch must be ascribed to the contracted smooth muscles it is difficult to ascribe the increased wall stiffness in SHR to any other factor than an increased bulk of contractile elements To this comes the fact that also the maximal contractile strength of the SHR resistance vessels has been shown to be considerably increased (Folkow *et al* 1970) also supported by the different levels of yield pressure observed in the present experiments (Fig 2 lower part) Both these findings strongly suggest the presence of an increased bulk of media as related to a somewhat restricted lumen together resulting in a considerable structurally determined increase in w/r ratio of the SHR resistance vessels Moreover the present method for estimating the circumferential distension of the resistance vessels tends to exaggerate its extent and the more so the higher the w/r ratio (see Results ) In other words the true difference in media stiffness and thickness between SHR—NCR may even be larger than indicated by the present measurements of wall distensibility from the r changes The indirect estimations of wall changes also in r or in $\frac{r_i+r_e}{2}$ briefly described strongly suggest that the media stiffness really is greater than the changes in $r_i$ would suggest and the more so the thicker the media

The combined evidence from these and earlier results (*cf* Folkow *et al* 1970 1971) further suggests that the structural w/r increase of the SHR resistance vessels is like the hyperthrophy of the left heart largely proportional in extent to the SHR—NCR difference in resting arterial pressure As also outlined earlier it will haemodynamically imply that the raised flow resistance in SHR can be maintained at an essentially normal level of smooth muscle activity in the resting steady state

This by no means denies that functionally induced exacerbations of vascular tone may particularly often occur in the hypertensive animals. It follows, however, that most current theories concerning the background of the chronically raised resistance in established primary hypertension have to be reconsidered.

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# Urinary Excretion of Noradrenaline after Treatment with α-Methyldopa Inhibition by a Central Nervous Mechanism

By

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## Abstract

ANDEN N F and M HENNING *Urinary excretion of noradrenaline after treatment with a methyldopa Inhibition by a central nervous mechanism* Acta physiol scand 1974 *90* 69—72

Normal rats treated with *L* α methyldopa (400 mg/kg i p ) after inhibition of the peripheral *L*-DOPA decarboxylase by α methyldopa hydrazine excreted significantly less noradrenaline in the urine The adrenaline excretion was only insignificantly reduced The results indicate that treatment with α methyldopa can inhibit the release of noradrenaline from the sympathetic nerves by decreasing the impulse flow from the central nervous system The adrenal medulla is influenced to a smaller degree

Treatment with α methyldopa can lower the normal and the elevated arterial blood pressure both in man and in animals (Oates *et al* 1960 Goldberg *et al* 1960 Henning 1967) The hypotensive effect is not due to the amino acid as such but to its amine metabolites α methyldopamine α methylnoradrenaline or both since it is abolished after inhibition of both the central and peripheral *L* DOPA decarboxylase activity (Davis *et al* 1963 Henning 1969) The mode of the hypotensive action of these α methylated amines is not completely understood (for review see Muscholl 1972) They can produce hypotension by a central nervous mechanism since treatment with α methyldopa does induce hypotension also when the *L* DOPA decarboxylase activity is selectively inhibited in the periphery (Henning 1969) In order to study if this α methyldopa induced hypotension is mediated via the sympathetic nervous system the urinary excretion of noradrenaline (NA) was determined in rats treated with α methyldopa in combination with a peripheral *L* DOPA decarboxylase inhibitor α methyldopa hydrazine The urinary excretion of free NA can be regarded as a measure of the total release of NA from the sympathetic neurons (Euler 1956)

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Acta physiol scand 1974 90 73—78

From the Department of Zoophysiology University of Uppsala, Sweden

# Absorption from the Ambient Water and Combustion of Ethanol in Young Atlantic Salmon (*Salmo salar* L ) at Different Temperatures

By

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## Abstract

HOGLUND L B L PILSTROM and M SJOBLOM *Absorption from the ambient water and combustion of ethanol in young Atlantic salmon (Salmo salar L ) at different temperatures* Acta physiol scand 1974 *90* 73—78

After 5—10 h exposure in static bio-assays first-summer *Salmo salar* live in equilibrium with the ambient ethanol concentration of 117 mmol/l at 10 and 16 C At 4 C this equilibration is retarded and is not evident until after 24 h A quantitative connection between ethanol oxidation (M) and body weight (W) in accordance with the general formula $M = K \times W$ seems to exist for young salmon at 16 C From the present tests the numerical values of the constants K and b were estimated to be approximately 10 5 and — 1 03 respectively Changes in behaviour induced by ethanol and the influence of temperature upon the metabolism of ethanol are discussed briefly


Ethanol is commonly used as a solvent *e g* in fish toxicological and/or fish physiological experiments Considerable amounts of ethanol may appear in waste water discharged from the manufactures of organic chemical compounds and pharmaceuticals Comparatively little is known however about the *absorption*[1] and *combustion* of ethanol by fish Ryback *et al* (1969) showed in static bio assays that gold fish live in equilibrium with the ambient ethanol solution at 21 8° C The present study deals with similar experiments on juvenile *Salmo salar* Conplementary tests have been made in an attempt to determine how the ambient temperature acts upon the absorption and the elimination of ethanol in relation to the medium by the latter species Experiments were run at 4° 10° and 16° C At 16° C the connection between body weight and the oxidation of ethanol by the fish was also studied The equilibrium between ethanol in the blood and in the surrounding medium is explained by the fact that ethanol is mainly dissolved in the water phase of the body fluids (*cf* Walgren *et al* 1970 p 35) In addition some observations on changes in

[1] The term *absorption* is used in the present study without any references to possibl p ocesses involved

TABLE 1 Chemical characteristics of the aerated test water during the experimental period based on several determinations The high pH is due to the aeration of this water rich in $HCO_3^-$

| pH | Oxygen tension as percentage of air saturation | Alkalinity expressed as $HCO_3^-$ in mmol/l |
|---|---|---|
| *8 0–8 3* | *85–100* | 4 75–5 11 |

behaviour during the stay in 117 mmol/l ethanol solution are reported For literature concerning the acute toxicity of ethanol to fish the reader is referred to the Water Quality Criteria Data Books issued by Battelle's Columbus Laboratories (1971)

## Materials and Methods

*Test fish and preacclimation*
Ninety first summer parr of *Salmo salar L* with a mean body weight of 6 0±0 2 g (range 2 8—10 3 g) and a length of 8 1±0 1 cm (range 6 5—10 1 cm) were used in the experiments. The fish were supplied by the Fishery Board from the breeding plant and salmon hatchery in Älvkarleby. They were kept in our laboratory in a 150 l aquarium with running aerated tap water (11—14 °C) No fish died either during the transport or later They were fed daily with EWOS salmon starting food size 1

*Water quality*
The tap water in Uppsala is subsoil water deriving from an esker in the area This water has a *fairly constant composition and a high ion content* The main constituents are Ca 2 0—2 2 mmol/l Mg 0 5—0 6 mmol/l and $HCO_3$ about 5 0 mmol/l See also Höglund (1961 Table 3 p 33 and Marcström (1967 Table 7 p 457) The tap water had to be aerated during the experiments and the water quality was examined regularly during the experimental period Oxygen was determined by ordinary Winkler titration pH with the aid of a Beckman pH meter type 22 and the total alkalinity according to Berger (*cf* Karlgren 1962) The results are presented in Table 1

*Test technique*
1 *Absorption experiments* In the static tests performed at 4 °C 10 °C and 16 °C 10 fishes were placed in 15 l tanks to be acclimated for two days and then transferred into identical tanks containing 117 mmol ethanol/l One fish was taken for blood ethanol determination after 1 2 4 8 12 24 36 48 and 60 h At the same time samples of the test medium were taken for analysis of the ethanol content Though the watery medium was continuously aerated the ethanol concentration did not change significantly The mean ethanol content in the water after 60 hours was 113 (range 111—115) mmol/l

2 *Ethanol determination in the blood* The fish were stunned with a blow on the head and the caudal fin was cut off Blood samples were drawn from the dorsal aorta into 25 µl heparinized glass capillaries and deproteinized in glass tubes containing 0 5 ml 6 % perchloric acid (PCA) The PCA was neutralized with 1 M KOH using solid phenolphthalein as the indicator The tubes were centrifuged at 2000 r p m in an ordinary laboratory centrifuge for 10 min The clear supernatant was used for the measurements of the ethanol concentrations according to Dickinson and Dalziel (1967) The reaction mixture contained 200 mmol/l tris buffer (Sigma Chem Co) pH 9 50 1 0 mmol/l NAD (Sigma Chem Co) and 0 15 mg yeast alcohol dehydrogenase (Boehringer) in a final volume of 3 ml The blanks contained all reagents except ethanol From the difference in extinction at 340 nm before and after the addition of yeast alcohol dehydrogenase the ethanol concentration was calculated

3 *Ethanol metabolism tests* After 48 hours stay in the ethanol solution of 117 mmol/l one fish at a time was placed in a 1 l E flask containing 1 0 l pure aerated tap water Samples of the latter medium (approximately 4 ml) were taken after 0 0 5 1 1 5 2 3 4 6 8 and 12 h and analyzed for ethanol Deriving from the transferred fish (mouth and opercular cavities body fluid compartments and so on) the ethanol concentration in this new environment increased rapidly and a maximum was reached within 5 h The ethanol concentration in the water then decreased linearly due to metabolic oxidation by the fish In the meantime the evaporation of ethanol from the liquid phase due to the aeration was found in this case to

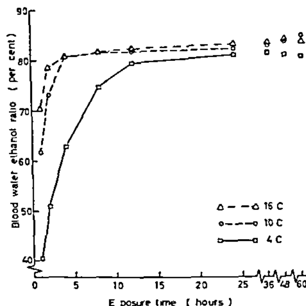



Fig 1 Blood ethanol levels in young *Salmo sala* expressed as percentage of the level in the medium at different temperatures plotted against exposure time Each dot represents the mean of corresponding determinations from three parallel tests The ethanol concentration of the medium varied between 111 and 117 mmol/l

affect the results Therefore in this type of experiment a correction for ethanol evaporation was made in the calculations of ethanol combus on by the fish which were based on the lopes of curves showing the elimination of ethanol from the surrounding medium Here also the ethanol concentrations were measured according to Dickinson and Dalziel (*op c t*)

## Results

### *Ethanol absorption*

The ethanol concentrations found in the fish blood after various periods of expo sure are given in Fig 1 as percentages of those in the water Each dot represents the mean value from three different determinations at each temperature *ii* 4° C 10° C and 16° C

The results of the absorption experiments performed at 10° C and 16° C show only minor deviations In both cases the ethanol concentration in the blood increased to 81—82 % within 5—10 h at which level it remained fairly constant throughout the last 50 h of these experiments The rate of ethanol absorption by the blood at 4° C was found to be considerably slower A concentration of 81 % was reached after approximately 24 h and this level showed very little alteration during the rest of the experimental period

### *Ethanol metabolism*

Six ethanol combustion tests on one single fish at a time were performed at 16° C In addition one of these tests was performed at 10° C and two at 4° C The re ults are shown in Fig 2 In the ob ervation material from the tests at 16° C there seems to be a rectilinear relationship between the logarithm of the rate of ethanol con

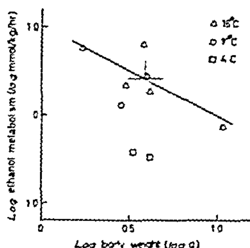



Fig 2 Relationship between the rate of ethanol metabolism and log body weight in young *Salmo salar* after 48 h stay in a 117 mmol/l ethanol solution at 16 C Single fish were transferred to flasks containing pure aerated Uppsala water at 16 C The coefficient of correlation is −0.78 The equation of the regression line log M = −1.03 log W − 1.02 Three estimates from similar tests at 10 and 4 C are also given.

sumption (M in mmol/kg body weight/h) and the logarithm of the body weight of the fish (W in g) ($r = -0.78$ $n = 6$ $0.1 > P > 0.05$*)

*Behavioural observations*
After a while certain behavioural reactions were initiated which seemed to be typical for salmon parr exposed to 117 mmol/l of ethanol in the water

## Discussion

*Absorption and metabolism*
Ryback *et al* (1969) reported that goldfish live in equilibrium with the ethanol concentrations of 87—141 mmol/l in the ambient water at 21.8 C The blood ethanol level was approximately 83 % of that in the medium after three hours and remained at this level for 5 days These results are in essential agreement with those obtained in the present study for salmon parr at 4, 10 and 16° C This might be explained by the fact that fish blood contains approximately 83 % water and only about 4 % of the ethanol is distributed to the lipid phase (Harger *et al* 1956) The minor deviations in the results from the two studies on fish may be explainable by small differences in the composition of the blood. No exact data on the blood water content of the goldfish and of young *Salmo salar* seem to exist However the figure for the rainbow trout (*Salmo gairdneri*) is 86 % (Black *et al* 1962) for mammals 81—87 % and for the chicken 87 % (Spector 1956) The rate of passive diffusion of ethanol into the fish apparently is faster than the rate of ethanol metabolism. It appears from Fig 2 that the metabolism of ethanol increases with rising temperature possibly corresponding to a $Q_{10}$ of about 2.3 in accordance with van t Hoff's rule It is reasonable to assume that the absorptions recorded in Fig 1 are mainly due to physical processes including diffusion the activity rates of which rise with increasing temperature corresponding to a lower $Q_{10}$.

A decrease in temperature from 10° to 4° C, however has a greater retarding effect on the rate of equilibration than a decrease in temperature from 16° to 10° C This retardation can hardly be explained by an assumption of underlying fairly constant temperature coefficients or $Q_{10}$ values Instead the existence of some kind of threshold effect within the former temperature range is indicated This may be due to an increased viscosity of the blood and a lower cardiac output (v Buddenbrock 1967 p 183) thus also reducing the rate of exchange of ethanol between water and blood

The relationship log M = b log W + log K is transformed to M = K×W As estimated from the slope of and the intercept on the Y axis of the regression line in Fig 2 we arrive at the relationship $M = 10\,5 \times W^{1\,03}$ between ethanol combustion in mmol/h/kg body weight at 16° C and body weight in g of first summer *Salmo salar* parr

*Behaviour*

Ryback (1969) reported that an ethanol concentration of 87 mmol/l in the water facilitated whereas 130 mmol/l greatly retarded learning by goldfish in a maze In our study the young salmon usually assumed resting positions on the bottom which is normal when encountering favourable environmental conditions (Kalleberg 1958 Hoglund 1961 Sprague 1968) After a few hours exposure to 113—117 mmol/l of ethanol they begin to swim around vividly Very often a fish is observed with its head near the surface and with the long axis of the body in an almost vertical position gulping air and intensively lurching by restless propulsions with their caudal fins while the pectoral fins are stiffly extended Similar behavioural changes are provoked among Atlantic salmon parr by for example moderately high carbon dioxide tensions and certain anesthetics added to the water (*cf* Hoglund and Borjeson 1971 Hoglund and Persson 1971)

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# An Autoradiographic Study of the Intestinal Absorption of ²Na

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## Abstract

JODAL M *An autoradiographic study of the intestinal absorption of ² Na* Acta physiol scand 1974 *90* 79—85

The distribution of ² Na within the villous tissue was studied by means of an autoradiographic technique during absorption of different saline solutions containing the tracer the resolving power being about 10 μm After the first minute of absorption most of the grains were localized in the epithelial cells the grain density in the lamina propria being low The same distribution of ²²Na was seen after 5 min incubation during maximal vasodilatation and during absorption of a hypotonic saline solution On the other hand a constant intraluminal perfusion of isotonic saline for 30 min changed the autoradiographic picture the highest radioactivity concentration now being found in the lamina propria. The gradient of grain density along the villous length was however not as marked as that reported by Haljamae *et al* (1973) who proposed that this gradient was created by a countercurrent multiplication of sodium in the hairpin vascular loops in the villi The results in this paper support the hypothesis that both sodium and water are crossdiffusing in such a system between the vessels constituting the countercurrent exchanger

In a previous publication (Haljamae Jodal and Lundgren 1973) the sodium concentration in the intestinal villous tissue expressed as amount of sodium per unit weight tissue protein was reported to be about four times higher at the tip than at the villous base during absorption of isotonic saline at resting intestinal blood flow On the basis of several different types of experiments it was proposed that this marked sodium concentration gradient along the villous length was explained by the presence of a countercurrent multiplication of sodium in the villi the exchanger being constituted by the villous central artery and the dense subepithelial capillary network A small increase of the plasma sodium concentration in the villous capillaries caused by an active absorption of this solute (below called single effect *cf* Dicker 1970) would result in a crossdiffusion either of sodium from the capillary network into the central artery or/and of water in the opposite direction In both cases the central artery will deliver a plasma to the villous tip of increased sodium concentration Hence the multiplication of the single effect in the countercurrent

exchanger will lead to a sodium concentration gradient along the villous length (cf. the situation in the renal papillae) as found by Haljamäe *et al.* (1973). These authors furthermore proposed that the villous hyperosmolarity thus created constitutes a driving force that allows for water absorption even when the intestinal contents are iso- or hyperosmolar in relation to plasma.

In the present paper the unidirectional influx of the radioactive isotope $^{22}$Na into the intestinal mucosa was studied by means of an autoradiographic technique in order to explore whether sodium or water is the dominant crossdiffusion "particle" in the intestinal countercurrent exchanger. Furthermore, such a technique may make it possible to obtain evidence for the possible hyperosmolarity in the intercellular spaces between the epithelial cells proposed to exist by Schultz and Curran (1968) and Diamond (1968) in their models for intestinal water absorption.

## Methods

*A. Operative procedures.* The experiments were performed on 9 cats anesthetized with chloralose 50 mg/kg b.wt. after induction with ether. The animals had been deprived of food for at least 24 h and had no obvious signs of intestinal infection. The preparation was similar to that described earlier (Hallbäck, Jodal and Lundgren 1973). Briefly, 3—4 jejunal segments 3—10 cm in length were isolated and the remainder of the intestinal tract was extirpated. The total venous outflow via the superior mesenteric vein was measured by an optical drop recorder unit. Blood pressure was recorded from the right femoral artery by a pressure transducer. The splanchnic nerves to the intestinal segments were cut, while the vagal supply was left intact. Vasodilatation was induced by a constant infusion of isopropylnoradrenaline 5—10 µg/min into the superior mesenteric artery.

*B. Test solutions.* The following saline solutions were used in the present study: 150 mEq NaCl/l, 135 mEq NaCl/l + 30 mmol glucose/l or 75 mEq NaCl/l (osmolarity around 290, 290 and 140 mOsm/l, respectively). The solution to be used was administered into the intestinal segment in two ways. In some experiments 0.5—1 ml was placed in the gut lumen for a predetermined time. The solutions used in this way were labelled with the radioactive sodium isotope $^{22}$Na (Radiochemical Centre, Amersham, England, specific activity 100 mCi/mg), the concentration of $^{22}$Na being 20 µCi/ml in the isotonic solution and 10 µCi/ml in the hypotonic one. In other experiments the intestinal segment was perfused at a constant rate of 0.4 ml/min for 30 min, thus assuring a fairly constant intraluminal sodium concentration. The concentration of $^{22}$Na in the perfusate was 5 µCi/ml. The intraluminal temperature of the experimental segments were continuously controlled by a thermocouple thermometer (Elektrolab, Copenhagen).

*C. Autoradiographic technique.* The technique has been described in detail by Haglund, Jodal and Lundgren (1973) and will only briefly be outlined here. At a predetermined time the intestinal segment was emptied by gentle squeezing, rapidly extirpated and immediately frozen in isopentane precooled by liquid nitrogen, a procedure which took about 1—2 s. The intestinal specimens were allowed up to 4 h equilibration to the cryostate temperature (-20 to -25 °C), after which time they were cut transversely in 3—5 µm thick slices in dark room. The tissue sections were adhered to emulsion coated slides (Ilford K 2) warmed to -5 °C with a light touch (Appleton 1966, Haglund, Jodal and Lundgren 1973). The autoradiographs were exposed at -20 °C together with a drying agent (Silica gel) for varying periods of time (3—12 weeks). Before developing the autoradiographs they were allowed to equilibrate with room temperature for 1 h. The tissue sections were then fixed in 5 % acetic ethanol, developed in Phen-X® (Ilford) for ? min, fixed for 5 min in Ilofix® (Ilford) and rinsed in distilled water. The sections were stained with hematoxylin-eosin and mounted in DePeX®.

Control non-radioactive tissue sections gave no chemographic effects on the emulsion with the technique used. Autoradiographs exposed at -80 °C gave the same result as those exposed at -20 °C. With the method used (thickness of emulsion 2—3 µm, thickness of tissue slices 3—5 µm) the resolving power of the autoradiographs was probably about 10 µm (Appleton 1966; see discussion).







Fig 1 Fig 2

Fig 1 The distribution of Na within a villous tip after half a minute absorption of isotonic saline containing the tracer Most of the grains are seen as large pale spots Blood flow 40 ml/min × 100 g Arrows indicat the epithelial cell borders L = lumen LP = lamina propria n = nucleus of the epithelial cell Exposure time 29 days Hematoxyline eosin Phase contrast microscopy 465 ×

Fig 2 The distribution of $^{22}$Na within a villous tip after 5 min absorption of isotonic saline containing the tracer Blood flow 26 ml/min × 100 g Arrows indicate the epithelial cell borders L = lumen LP = lamina propria n = nucleus of epithelial cell Exposure time 48 days Hematoxyline eosin Phase contrast microscopy 440 ×

## Results

Fig 1 illustrates the distribution of absorbed Na within a villous tip after half a minute exposure of the intestinal mucosa to isotonic saline containing the tracer during resting blood flow (40 ml/min × 100 g) Most of the grains obtained with Na were *comparatively* large and photographed with a *phase contrast microscope* these large grains are seen as pale spots in Fig 1 and the following Fig Most of the radioactivity was in Fig 1 found in or close to the epithelial cells with only a few grains in the lamina propria When the incubation period was prolonged to 5 or 15







Fig 3a Fig 3b

Fig 3 The distribution of $^{22}$Na within a transection of a villous tip (left panel) and along the villous length (right panel) after a constant intraluminal perfusion for 30 min of an isotonic saline solution Blood flow 29 and 21 ml/min × 100 g respectively Arrows indicate the epithelial cell borders CL = filled central lymphatic lacteal L = luminal gap between two adjacent villi LP = lamina propria Exposure time 57 and 36 days respectively Hematoxyline-eosin Phase contrast microscopy 515 × and 315 × respectively

min the autoradiographic picture was markedly changed (Fig 2) the largest grain concentration being now localized to the lamina propria where the grains are diffusely localized without concentration to any particular histological structure

To ascertain a constant intraluminal concentration of $^{22}$Na during longer incubation periods the tracer solution was continuously perfused through the intestinal lumen for 30 min The villous localization of absorbed $^{22}$Na in such an experiment is illustrated in Fig 3 The left panel of this Fig shows the distribution of radioactivity of a cross section of a villous tip The distribution pattern of grains is about the same as in Fig 2 i e the highest grain concentration is found in the lamina propria and within the wellfilled central lymphatic lacteal The grain density in the lamina propria seemed however seldom to exceed twice that found over the epithelial cells




Fig 4 Transection of a villous tip showing the distribution of $^{22}$Na within the villous tissue after 5 min absorption of hypotonic saline (140 mOsml/l) containing the tracer Blood flow 26 ml/min × 100 g Arrows indicate the epithelial cell borders L = lumen LP = lamina propria n = nucleus of epithelial cell Exposure time 57 days Hematoxyline eosin Phase contrast microscopy 530 ×

A gradient of grain density along the villous length in its top half was often observed in the present study as is illustrated in Fig 3 right panel It is evident from this figure that the number of grains per area is greater at the tip than at the base of the illustrated villus *i e* 350 µm from the tip This ratio of grain density between the tip and the base half down the villous length was at most 2 1

The autoradiographs of Fig 1—3 are also representative for experiments in which the tracer solution contained glucose a solute known to enhance the rate of sodium absorption However when the incubation medium was changed from an isotonic to a hypotonic saline solution (75 mEq NaCl/l) the autoradiographic picture was markedly changed as is shown in Fig 4 obtained after 5 min absorption at a resting blood flow of 26 ml/min × 100 g The grains were then almost exclusively located to the epithelial cell fraction of the villus

Maximal vasodilatation (mean blood flow 255 ml/min × 100 g range 205—280) induced by i a infusion of isopropylnoradrenaline seemed to markedly decrease the number of grains in the villous core as compared to the situation at rest (Fig 2 and 3) and the resulting autoradiographs were similar to that of Fig 4

## Discussion

The present study represents an attempt to study the absorption of sodium from the intestinal lumen with an autoradiagraphic technique using $^{22}$Na This radioactive sodium isotope emits $\beta$ radiation with a mean energy level of 193 Kev (Quimby and

Feitelberg 1963) which should be compared to the corresponding value for $^{3}H$ being 6 Kev This pronounced difference in β-energy levels implies that the autoradiographic resolving power of $^{22}Na$ is much less than for $^{3}H$ Appleton (1966) examined the resolving power when using $^{22}Na$ with an autoradiographic technique similar to the present one and then reached a value of 9—13 μm This figure thus represents the distance from a point source to the point where the grain density is reduced to half the value observed over the source In this study the tissue was frozen within 1—2 s after extirpating the organ which probably means a significantly shorter fixation time than used by Appleton and consequently a smaller diffusion artefact Furthermore the thickness of the tissue section and the photographic emulsion layer was somewhat larger in the work of Appleton while the time of exposure was shorter in Appleton s experiments Thus it seems justified to conclude that the resolving power in the present study probably is 10 μm at most It is therefore not possible to localize the grains in the autoradiographs to the extra or intracellular spaces in the villus with any high degree of accuracy

However by comparing the autoradiographs obtained after various times of exposure to the labelled solutions it was possible to follow the absorptive path of the tracer Thus a comparatively small number of grains were found in the central portion of the lamina propria after 30—60 s exposure to the labelled solution (Fig 1) This observation seems to indicate that a diffusion barrier exists at the tissue" side of the epithelial cells represented by either the plasma membrane and/or the capillary network The observation that a similar distribution pattern of grains were observed even after longer exposure times when absorption occurred during maximal vasodilatation or from a hypotonic saline solution (Fig 4) may suggest that the capillaries constituted the hindrance to diffusion Intense vasodilatation increasing the transport capacity of the villous blood flow easily washed out the absorbed $^{22}Na$ During hypotonic absorption on the other hand the amount of transported $^{22}Na$ was so markedly reduced (cf Vaughan 1960 Grim 1962 Annegers and Wakefield 1962) so that even the "resting blood flow evidently was able to effectively hinder net diffusion of the tracer into the villous lamina propria

However within 5 min of exposure at a resting blood flow the tracer content in the villous core had increased considerably (Fig 2 and 3) suggesting a diffusion of the absorbed tracer beyond the blood stream in the villous capillaries into the stroma. However the countercurrent mechanism may also be involved as discussed in the introduction According to this hypothesis the absorbed sodium increasing the plasma concentration of the solute in the subepithelial capillaries ("single effect would diffuse from the capillary network into the villous central artery to be brought back again towards the villous tip If such a countercurrent exchange diffusion of the absorbed $^{22}Na$ occurred one would expect to find a multiplication of the single effect leading to a grain density gradient along the villous length Such a gradient was also found the ratio between tip and base amounting to at most 2 1 This ratio should be compared to the corresponding ratio of 3—4 1 observed in the previous publication where the total content of sodium was determined in tissue

sections cut perpendicularly to the long axis of the villi The discrepancy between these two tip to-base ratios is probably explained by a countercurrent transfer of water from the villous central artery to the subepithelial capillary network induced by the increased plasma osmolarity in the capillaries Thus in conclusion one may state that the countercurrent multiplication of sodium in the intestinal villi occurring during absorption of sodium chloride is partly caused by an exchange diffusion of sodium partly by an exchange transfer of water

It has been proposed, as mentioned in the introduction that the intercellular spaces surrounding the epithelial cells contains the hyperosmolar solution necessary to explain intestinal water absorption against a concentration gradient (Diamond 1968 Schultz and Curran 1968) With the relatively low resolving power of the present autoradiographic technique (see above) it was not possible to determine the grain density within these narrow intercellular spaces not even at their wider basal parts On the other hand one would expect that an increased intercellular sodium concentration to its major part would be created by sodium ions just absorbed from the lumen *i e* with a high content of Na as compared to the surrounding tissue As seen in Fig 2 the grain density is neither in the epithelial cells nor in the basal half of these cells higher than in the adjacent villous core Thus this study provides no support for a regional hyperosmolarity within the intercellular spaces but conclusive evidence cannot be obtained until better techniques are available *e g* electronmicroscope autoradiography for soluble substances

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Acta physiol scand 1974 90 86—93

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# Prostaglandin Action on Noradrenaline Release and Mechanical Responses in the Stimulated Guinea Pig Vas Deferens

By

PER HEDQVIST

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## Abstract

HEDQVIST P *Prostaglandin action on noradrenaline release and mechanical responses in the stimulated guinea pig vas deferens* Acta physiol scand 1974 90 86—93

$PGE_1$ and $PGE_2$ reversibly and dose-dependently inhibited transmitter release from the stimulated guinea pig vas deferens. The two compounds proved equiactive and more prone to inhibit transmitter release at a low than at a high stimulation frequency. The PGEs inhibited the twitch response but produced inconsistent effects on contractions induced by prolonged stimulation. The difference may be due to the fact that the PGEs reduce the excitatory junction potentials and depolarize the effector cell membrane. Phenoxybenzamine increased transmitter release in response to nerve stimulation but failed to significantly alter the inhibitory effect of $PGE_2$. It is therefore concluded that PGEs and presumed presynaptic α receptors probably intrude upon different levels of the process of excitation secretion coupling. A possible model for the action of locally formed PGEs on the neuromuscular transmission of the vas deferens is advanced

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Prostaglandins of the E type ($PGE_1$, $PGE_2$) are known to inhibit transmitter release in response to sympathetic nerve stimulation in the cat spleen and the rabbit heart (Hedqvist and Brundin 1969 Hedqvist 1970 Hedqvist and Wennmalm 1971) Indirect evidence for the same type of prejunctional action has been presented using the hindleg of the cat and the dog and the seminal vesicle of the guinea pig (Kadowitz *et al* 1971 Hedqvist 1972 a b) More divergent results have been obtained when the effect of the PGEs has been studied on the motor innervation of the guinea pig vas deferens Thus PGEs have been reported either to potentiate the neuroeffector transmission (Mantegazza and Naimzada 1967 Bhagat *et al* 1972) or to inhibit the effector response to nerve stimulation while potentiating that to noradrenaline (NA) (Euler and Hedqvist 1969 Ambache and Zar 1970 Hedqvist and Euler 1972)

TABLE I Cation exchange column chromatography of radioactivity in effluent from superfused guinea pig vas deferens previously loaded with $^3$H NA Chromatographic values presented as relative distribution (per cent) of not adsorbed material ( acids ) intact $^3$H NA and H normetanephrine ($^3$H NM) Means±S E figures within brackets = number of expts

| Chromatogr product | Prestimulation | During stimulation |
|---|---|---|
| Acids | 83 8±1 7 | 27 4±6 0 |
| $^3$H NA | 15 2±1 5 (3) | 71 9±6 0 (6) |
| $^3$H NM | 0 5±0 3 | 0 3±0 2 |

In the present study the effect of PGEs on transmitter release and mechanical responses to nerve stimulation in the guinea pig vas deferens is analyzed in order to obtain more definite information about their influence on the neuroeffector transmission A preliminary account on part of the results has been presented elsewhere (Hedqvist 1972 c)

## Methods

The experiments were conducted on male guinea pigs weighing 500—700 g The animals were killed by a blow on the head and the vasa deferentia were isolated and carefully dissected free from adjacent tissue The isolated preparation was incubated for 1 5 in Tyrode s solution containing 25 μCi/ml of $^3$H l NA (spec. act 5 4 Ci/mmol) and was then thoroughly rinsed and continuously superfused in a 2 ml organ bath with NA free Tyrode at a rate of 1 ml/min The composition of the solution was (concentrations in mM) NaCl 136 7 KCl 2 7 $CaCl_2$ 1 8 $MgCl_2$ 0 5 $NaHCO_3$ 11 9 $NaH_2PO_4$ 0 4 glucose 5 5 ascorbic acid 0 1 The solution was kept at 37 C and bubbled with 5 % $CO_2$ in $O_2$ The preparation was electrically stimulated by means of platinum electrodes in the wall of the bath and a Grass S4 stimulator delivering trains of pulses (5—10 Hz 1 ms duration supramaximal voltage) at 10—20 min intervals Contractions of the organ were recorded isotonically The radioactivity in superfusate samples and extracts of organ homogenates was separated in that of intact NA and that of its metabolites by cation exchange column chromatography (amberlite SG 120) The samples were put on the column at pH 6 and fractional separation was performed with acetate buffer pH 6 distilled water and increasing contractions of hydrochloric acid (0 5—2 0 M) The recovery of authentic NA added to the samples and carried through the entire chromatographic procedure was 85 9±0 9 per cent (mean±S E n = 6) The radioactivity of the different superfusate samples and organ extracts was determined by counting 0 1—1 0 ml aliquots in a Packard liquid scintillation spectrometer As counting medium was used 10 ml Instagel or 20 ml of a 3 7 ethanol toluene mixture containing 4 g PPO and 0 1 g POPOP per liter of toluene Quenching was monitored by internal standards

$PGE_1$ and $PGE_2$ were generously supplied by Dr J Pike Upjohn Company Kalamazoo U S.A and phenoxybenzamine (Dibenzyline) by Smith Kline and French Welwyn City England

## Results

Transmural stimulation of the guinea pig vas deferens preincubated with $^3$H NA caused increased efflux of radioactivity of which 72 per cent was accounted for by intact $^3$H NA while during resting periods $^3$H NA represented 15 per cent of the radioactivity appearing in the effluent from the organ (Table I) Independently whether the preparation was stimulated or not the efflux of $^3$H normet

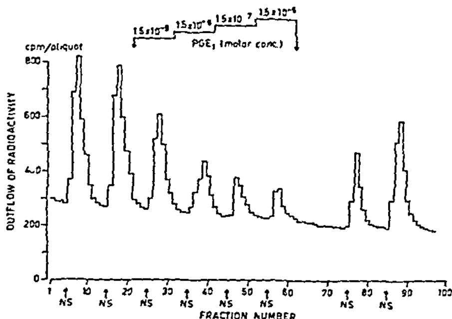



Fig 1 Guinea pig vas deferens previously loaded with $^3$H NA. Effect of increasing doses of $PGE_1$ on outflow of tracer in response to transmural stimulation (NS) 450 pulses at 5 Hz. Time in min = fraction numbers

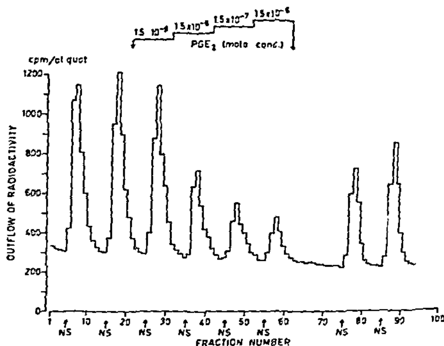



Fig 2 Guinea pig vas deferens previously loaded with $^3$H NA. Effect of increasing doses of $PGE_2$ on outflow of tracer in response to transmural stimulation (NS) 450 pulses at 5 Hz Time in min = fraction numbers

TABLE II Mechanical response (contraction amplitude and area) and efflux of ³H radioactivity from guinea pig vas deferens stimulated at 5 Hz in the presence of $PGE_1$ (0 5—500 ng/ml) Values presented as per cent of figures obtained during control stimulation Means ± S E

| Number of expts | $PGE_1$ dose ng/ml | % of control stim | | |
|---|---|---|---|---|
| | | H efflux | Contr ampl | Contr area |
| 6 | 0 5 | 77±9* | 99±1 | 95±12 |
| 3 | 1 | 54±10 | 85±16 | 90±18 |
| 8 | 5 | 35±6*** | 79±12 | 75±14 |
| 7 | 10 | 30±6 ** | 62±21 | 60±13* |
| 6 | 50 | 18±3* * | 85±7 | 85±11 |
| 8 | 500 | 15±4*** | 95±7 | 96±10 |

* $P < 0 05$ ** $P < 0 001$

TABLE III Mechanical response (contraction amplitude and area) and efflux of ³H radioactivity from guinea pig vas deferens stimulated at 10 Hz in the presence of PGE and PGE (10 ng/ml) Values presented as per cent of figures obtained during control stimulation Means ± S E

| Number of expts | Drug | of control stim | | |
|---|---|---|---|---|
| | | H NA | Contr ampl | Contr area |
| 8 | $PGE_1$ 10 ng/ml | 45±3*** | 100±2 | 91±6 |
| 8 | $PGE_2$ 10 ng/ml | 49±4 * | 110±10 | 130±10 |

* $P < 0 001$

was always very low The vast majority of the radioactivity not identified as ³H NA consisted of material not adsorbed on the amberlite column and therefore probably represented deaminated products (*cf* Langer 1970)

Over a wide range of doses $PGE_1$ and PGE consistently and reversibly inhibited the efflux of ³H NA from the transmurally stimulated vas deferens (Fig 1 and 2) Inhibition was seen with as little as 0 5 ng/ml and progressively increased with the dose (Table II) There was no difference in effect between the two compounds but they were less effective in inhibiting ³H NA release at 10 Hz than at 5 Hz stimulation (significant at the 5 per cent level by Student s t test for paired variates) (Table III)

The twitch response to transmural stimulation (25 pulses 5—10 Hz) was consistently inhibited by the PGEs confirming previous observations (Hedqvist and Euler 1972) Concerning more prolonged stimulation at 5 Hz (300—450 pulses) the mean values for the effector responses decreased below the control level in the presence of low PGE doses and increased again with high doses (Table II) H

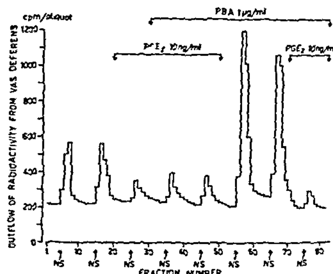



Fig 3 Guinea pig vas deferens previously loaded with $^3$H NA Effect of PGE$_2$ and PBA on outflow of tracer in response to transmural stimulation (NS) 450 pulses at 5 Hz. Time in min = fraction numbers

ever the effects were inconsistent varying from one experiment to the other from marked inhibition to unchanged or even potentiated response Therefore no statistically significant change of the mechanical response was seen at any dose possibly with the exception of 10 ng/ml PGE$_2$ ($p < 0.05$) However when the stimulation frequency was increased to 10 Hz no inhibition of the effector response was seen after 10 ng/ml PGE$_2$ (Table III)

Phenoxybenzamine (PBA) markedly increased the efflux of $^3$H NA to transmural stimulation (Table IV) (cf Hughes 1972 Johnson et al 1971) and altered the effector response inasmuch as the contraction area was heavily depressed while the amplitude remained unchanged or even potentiated (cf Boyd et al 1960 Swedin 1971)

The potentiating effect of PBA on $^3$H NA release was completely abolished by PGE$_2$ and meanwhile the inhibitory action of PGE$_2$ on $^3$H NA release was significantly increased ($p < 0.001$) (Table IV Fig 3) In the presence of PBA the

TABLE IV Efflux of H NA from guinea pig vas deferens stimulated at 5 Hz in the presence of PGE$_2$ (10 ng ml) PBA (1 µg/ml) and PBA+PGE$_2$ Values expressed as per cent of output figures obtained during control stimulation and in the presence of PBA Means ± S E figures within brackets = number of observations All effects significant at the 0 1 level by Student's t test for paired variates

| Stimulated relative efflux of H NA (per cent) | | | |
|---|---|---|---|
| PGE$_2$ / control | PBA / control | PBA+PGE$_2$ / control | PBA+PGE$_2$ / PBA |
| 30±6 | 421±65 | 41±9 | 11±1 |
| (7) | (9) | (13) | (9) |

mechanical response to stimulation was either inhibited or potentiated by $PGE_2$. When the outflow of $^3H$ NA in response to stimulation was depressed in advance by PGE, PBA did not significantly alter the overflow response until the administration of PGE was terminated (Fig 3)

## Discussion

PGEs have been reported to potentiate the contraction of the guinea pig vas deferens in response to nerve and transmural stimulation (Mantegazza and Naimzada 1965 Bhagat *et al* 1972) Sjöstrand and Swedin (1968) found inconsistent effects with $PGE_1$ varying from slight inhibition to moderate potentiation

Using short term stimulation neuromuscular transmission in the guinea pig vas deferens is markedly and reversibly inhibited by low to moderate doses of $PGE_1$ and PGE but potentiated by high doses of the two compounds (Euler and Hedqvist 1969 Ambache and Zar 1970 Hedqvist and Euler 1972) On the other hand the effector response to NA is always potentiated by PGEs even in doses known to inhibit the mechanical response to nerve stimulation Therefore a dual action has been proposed prejunctional inhibition of transmitter release and postjunctional potentiation of the mechanical response to NA released (Hedqvist and Euler 1972) Further support for an inhibitory action on NA release rests on observations that PGEs inhibit the release of dopamine $\beta$ hydroxylase and depress excitatory junction potentials in the stimulated vas deferens (Johnson *et al* 1971 Sjöstrand 1972 Taylor and Einhorn 1972)

In the present study $PGE_1$ and PGE within a wide range of doses consistently and reversibly inhibited the efflux of $^3H$ NA and therefore in all probability the outflow of endogenous NA from the transmurally stimulated guinea pig vas deferens The inhibition progressively increased with the dose and showed no difference between the two compounds The effect was more marked when the organ was stimulated at 5 Hz than at 10 Hz confirming previous observations that the PGEs are more prone to inhibit neuroeffector transmission at low nerve activity than at high (Hedqvist and Euler 1972)

In contrast to transmitter efflux and twitch response the contractions to prolonged transmural stimulation were inconsistently affected by the PGEs possibly with the exception of 10 ng/ml of $PGE_2$ at a stimulation frequency of 5 Hz Since the stimulation parameters exclude direct stimulation of the effector cells (*cf* Hedqvist and Euler 1972) a probable explanation is that PGEs in spite of decreasing the excitatory junction potentials (due to inhibition of transmitter release) by depolarizing the effector cell membrane bring the potential closer to the firing level and thereby promote propagation of action potential (*cf* Sjostrand 1972) On the other hand the twitch response is probably inhibited because of a loss of time for the reduced excitatory junction potentials to reach by summation the firing level for initiation of propagated action potentials

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# Studies on the Accumulation of Noradrenaline and 5-Hydroxytryptamine by Cholesterol-Phospholipid Microvesicles

By

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## Abstract

AHTEE L and S M JOHNSON *Studies on the accumulation of noradrenaline and 5 hydroxytryptamine by cholesterol phospholipid microvesicles* Acta physiol scand 1974 90 94—106

A study was made of the accumulation of the monoamines 5 hydroxytryptamine and noradrenaline by phospholipid vesicles of similar chemical composition to the natural monoamine storage vesicles Although the artificial vesicles would accumulate the monoamines against a concentration gradient they were far inferior in this respect to the natural vesicles The accumulation of the monoamines was studied as a function of time temperature and the chemical composition of the vesicles and the effect of osmotic shock reserpine metaraminol and calcium on the release of the monoamines was noted The results indicated that the binding of 5 hydroxytryptamine was increased by acidic phospholipids but that noradrenaline was unaffected A temperature rise only marginally inreased the monoamine accumulation Reserpine slightly retarded the release of noradrenaline

In nature monoamines are stored in small structures bounded by phospholipid membranes such as nerve vesicles platelet storage granules or the chromaffin granules of the adrenal medulla Recently the preparation and structure of single bilayer phospholipid vesicles about 24 nm diameter has been described (Huang 1969 Johnson and Bangham 1969 Johnson et al 1971) When cholesterol is present the size of these vesicles increases (Johnson 1973 Johnson and Buttress 1973) and they approach the size of the natural storage vesicles

The binding of catecholamines to phospholipids has been investigated by Euler (1946) Norlander (1950) Cuthbert *et al* (1967) and Formby (1967 1968) All the e studies involved the use of a specific phospholipid to change the partition coefficient of a monoamine between an aqueous and organic phase Now however, the phospholipid composition and cholesterol/phospholipid molar ratios for various monoamine storage structures are known for example chromaffin granule membranes 0 58 (Blaschko *et al* 1967) platelet granules 0 61 (Marcus Ullman and Safier

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1969) synaptosomes 0 41 (Eichberg Whittaker and Dawson 1964) and nerve trunk vesicles 0 45 (Lagercrantz 1971 b)

In the present experiments we studied the accumulation of dl noradrenaline (NA) and 5 hydroxytryptamine (5HT) from aqueous solution by lipid bilayer microvesicles (lipo omes) with the approximate diameter of synaptic vesicles made of a cholesterol phospholipid mixture in which the proportion of cholesterol to phospholipid was 0 41 and the composition of the phospholipid mixture (see Methods) was made to mimic as well as possible the composition of synaptosomal phospholipids as described by Eichberg *et al* (1964)

## Material and Methods

The method of preparation of the phosphol pids is given by Papahadjopoulus and Miller (1967) Briefly phosphatidyl choline was extracted from egg yolks and phosphatidic acid prepared from it by enzymic hydrolysis Phosphatidyl ethanolamine was also extracted from egg yolks and phosphatidyl serine from beef brain The cholesterol was Sigmas chromatographic grade The dl noradrenaline HCl used to dilute the isotopic noradrenaline came from Sigma and 5 hyroxytryptamine creatinine sulphate with which isotopic 5HT was diluted from Fluka AG Buchs SG Switzerland The dl $^3$H noradrenaline hydrochloride $^{14}$C and $^3$H 5 hydroxytryptamine creatinine sulphate and $^{86}$RbCl were obtained from the Radiochemical Centre Amersham England Tris base (Sigma 7—9 buffer grade) was recrystallized from water then reagent grade was used All other reagents were analytical grade and the water was twice distilled The drugs studied were 1 metaraminol HCl (Merck Sharp & Dohme Rahway N J ) and reserpine (Ciba AG Basel) Reserpine was dissolved in 10 % ascorbic acid and diluted further with water Metaraminol was dissolved in KCl buffer

The aqueous solution used throughout these experiments and hereafter de cribed as KCl buffer contained 5 mM RbCl 100 mM KCl 50 mM Tris-HCl and was buffered at pH 7 3 (37 C) Unless otherwise stated the phospholipid mixture used was that given in Table I where it is compared to the phospholipid composition of natural storage vesicles The stock phospholipid cholesterol mixture was kept dissolved in chloroform under nitrogen at −20 C

TABLE I The compos t on of the standard phosphol pid mixtu e used compared to the composition of biological storage vesicles The molar ratio of cholesterol to phospholipid and the individual phospholipids as percentage of total lipid phosphorus are shown

| | Standard m xture | Synaptosomes (Eichberg *et al* 1964) | Adrenal medull ry vesicles (Blaschko *et al* 1967) | Nerve trunk vesicles (Lagercrantz 1971 a) | Platelet granules (Marcus *et al* 1969) |
|---|---|---|---|---|---|
| Cholesterol/ phosphol pid | 0 41 | 0 41 | 0 56 | 0 45 | 0 61 |
| PC | 53 2 | 39 3 | 26 0 | 36 6 ° | 31 8 ° |
| PE | 28 9 | 33 6 | 36 1 * | 30 8 ° | 28 7 * |
| PS | 16 2 | 16 9 | 9 2 ** | 7 1 °/** | 15 6 * |
| PA | 1 7 | 2 3 ** | 0 6 ** | 4 9 ° *** | |
| Lysolecithin | — | — | 16 8 | 3 1 ° | 1 9 |
| Sphingomyelin | | 5 3 | 10 9 | 17 6 | 20 2 |
| Recovery | | 97 4 | 99 6 | 100 1 | 98 2 |

PC = phosphat dylcholine PE = phosphat dylethanolam ne PS = phosphatidylser ne PA = phosphatid c acid

° Eth nol mine plasmalogen included * PS+phosphatidylinositol ** PA+cardiol pin

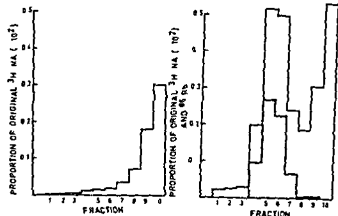



Fig 1 Histograms showing the distribution of $^{3}H$ noradrenaline ($^{3}H$ NA) in the eluate of Sephadex column Pillars represent the proportions of the original isotopes eluted in each 1 3 ml fraction after discarding the first 7 5 ml Results are mean of 3 expts The histogram on the left is $^{3}H$ NA only and the one on the right represents liposomes with $^{86}RbCl$ inside (only) incubated for 4 h with 10 M NA at 37 C NA fraction × 10 ■ $^{86}Rb$ □ $^{3}H$ NA

*Liposome preparation* In a typical experiment about 30 μmol of the mixed lipids in chloroform were evaporated to dryness under reduced pressure in a 100 ml round bottomed flask *Up to 1 ml KCl buffer containing $^{86}Rb$ was added the flask filled with nitrogen and gently* agitated until the lipids formed a thick milky suspension This was then transferred to a 1 3 cm diameter flat bottomed glass vial and sonicated under nitrogen at room temperature until the suspension became transparent (1/2—1 h) then stored overnight without openin at 4 C The sonicato was a Kerry's Ultrasonic cleaning bath type KB 80/1 operating at 80 kHz

The liposomes were separated from the untrapped isotope by passage over a 30 cm column of 3 g (anhydrous weight) coarse G 50 Sephadex as described by Papahadjopoulus and Watkins (1967) The 4 5 ml of KCl buffer containing the liposomes was diluted to 6 5 ml and two 1 ml portions were retained for counting Four other 1 ml portions were incubated at 37 C with the required concentrations of $^{14}C$ or $^{3}H$ 5 hydroxytryptamine (5HT) or $^{3}H$ noradrenaline (NA) in glass stoppered tubes in air Each portion was then put on the G 50 Sephadex column A typical isotope eluation pattern is shown in Fig 1 The first 11 ml were discarded the next 1 3 ml portions were stored separately in tubes and finally the column was washed free of isotope by about 100 ml KCl buffer 1 ml samples were taken from the 1 3 ml and put into Bray's solution (Bray 1960) $^{86}Rb$ and $^{3}H$ standards were prepared from 0 01 ml of the original solution added to the dry phospholipid ($^{86}Rb$) or 0 01 ml of the $^{3}H$ or $^{14}C$-isotope solution in which the liposomes were incubated 0 99 ml of KCl buffer was added to the standards then 10 ml Bray's solution A blank with 1 ml KCl buffer and 10 ml Bray's solution was also prepared The isotopic mixture was counted on a 2 channel Wallac Decem NTL314 liquid scintillation counter One channel was set to count low energy pulses ($^{3}H$ + some $^{86}Rb$) and the other high energy pulses ($^{86}Rb$) It was found that the phospholipid caused no additional quenching on either channel A correction for the $^{86}Rb$ was applied to the low energy channel and the following ratio calculated

$$\frac{\text{Count rate } ^{3}H \text{ in sample}}{\text{Count rate } ^{3}H \text{ in standard}} \times \frac{\text{Count rate } ^{86}Rb \text{ in standard}}{\text{Count rate } ^{86}Rb \text{ in sample}}$$

It was subsequently called the Na/Rb volume ratio This volume is a measure of the ability of liposomes to concentrate the monoamine from solution it does not give directly the amount of monoamine in the liposomes This can be found from volume ratio × Rb volume × monoamine concentration In some experiments two 0 5 ml samples were taken from the 1 3 ml collected from the Sephadex column In this case one was put into Bray's solution and counted for $^{14}C$ or $^{3}H$ (contaminated with $^{86}Rb$) and one was counted for $^{86}Rb$ only by its Cerenkov radiation in aqueous solution Appropriate blanks and standards were used

The remainder of the sample was analysed for lipid phosphorus by McClare's method (McClare 1971) or by the method of Kankare and Suovanniemi (1971) The volume inside the liposomes per μmol lipid phosphorus was calculated from the relation below assuming that the $^{86}RbCl$ is passively trapped rather than adsorbed by the liposome (Johnson and Buttress 1973)

$$\frac{\text{Count rate } ^{86}Rb \text{ in sample} \times 0.01}{\text{Count rate } ^{86}Rb \text{ in standard} \times \mu mol \text{ lipid P}}$$

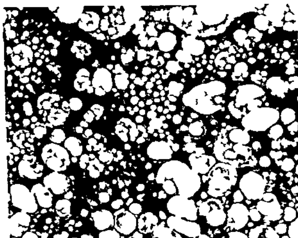


Fig 2 Electronmicrograph of highly sonicated liposomes prepared from the standard lipid mixture Negatively stained with 2 % phosphotungstic acid in aqueous solution Photograph was taken with Philips EM 200 Electron Microscope magnification 78 000 The diameter of the small vesicles was 22±5 7 nm (mean±S.D n = 24) (The electronmicrograph was taken by Dr E Solatunturi)

Due to the errors inherent in counting mixed isotope samples the Na/Rb volume ratio is accurate to ±10 % and the rubidium volume to ±5 %

Fig 2 is an electronmicrograph of a sonicated sample of the mixed lipid vesicles from which their mean size can be calculated The hollow bilayer nature of these structures is shown more clearly in Johnson *et al* (1971)

## Results

*Partition coefficient measurements*

The partition coefficients of NA and 5HT between KCl buffer and heptane were measured at 37° C Each sample was done in duplicate

$10^{-4}$ M and $5\times10^{-5}$ M NA in 2 ml KCl buffer were shaken with 2 ml heptane for 3 h at 37° C The two phases were separated by spinning in a Christ bench centrifuge for a few minutes Then 1 ml heptane phase+1 ml KCl buffer and 1 ml KCl buffer phase+1 ml heptane were counted in Bray s solution The ratio of the two count rates gave the partition coefficient 5HT was treated similarly The partition coefficient for NA in KCl buffer/heptane was $3\,0\pm0\,4\times10^3$ where ± is the S D (4 expts ) Similarly for 5HT the KCl buffer/heptane partition coefficient was $60\pm20\times10^3$ It should be noted that NA is 20 times more soluble in heptane than 5HT at pH 7 3 (37° C)

*Time course of the accumulation of NA and 5HT into liposomes*

Fig 3 shows the time course of the accumulation of NA and 5HT by liposomes made of the standard lipid mixture The final liposome suspension in the NA experiment contained 1 38 μmol phospholipid/ml The $^{86}$Rb volume of this suspension was 0 0018 ml = 1 3 μl μmol $P^{-1}$ In another time course experiment for NA uptake with the same phospholipid mixture the $^{86}$Rb volume was 0 0018 ml In the 5HT experiment the $^{86}$Rb volume of the final suspension was 0 0010 ml

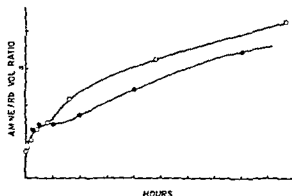



Fig 3 A comparative time course of the accumulation of noradrenaline and 5 hydroxytryptamine by liposomes at 37 C (Time scale for 5HT×1/5) ● 10 M NA O 10 M 5HT

Samples of these suspensions were incubated with NA (10 M) or with 5HT (10 M) for different lengths of time The accumulation of both amines by liposomes was positively correlated with the length of incubation The accumulation of NA was very fast during the first 15 min then it stayed at a plateau for about 1 to 2 h The NA/Rb volume ratio at 24 h was 50 and at 48 h 59 (these figures are corrected for the Rb lost during the incubation) The accumulation of 5HT into the lipo omes was 1/5 as fast as that of NA However when the time course for 5HT was corrected by this factor the initial accumulation was equal to that of NA and the later parts of the accumulation curves for these two amines were parallel The increase in absorption of both amines after the initial plateau region may be due to oxidation of the amines or the phospholipids or both or alternatively to a slow binding reaction between the monoamines and phospholipid

*Effect of the incubation temperature on the accumulation of NA and 5HT into liposomes*

Fig 4 shows the effect of the incubation temperature on the accumulation of NA by liposomes Liposomes were prepared from the standard lipid mixture The final liposome suspension had a $^{86}$Rb volume of 0 0033 ml in the 4 h expt the Rb volume

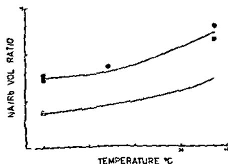



Fig 4 The accumulation of noradrenaline (10 M) by liposomes at different temperatures O 2 h incubation ● 4 h incubation

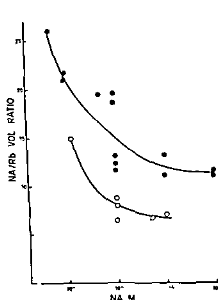



Fig 5 a

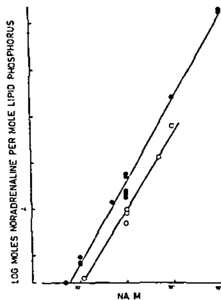



Fig 5 b

Fig 5 a The effect of the concentration of noradrenaline on its accumulation by liposomes at 37 C ○ 2 h incubation ● 4 h incubation
*Fig 5 b The moles of noradrenaline adsobrbed per mole of lipid phosphorus as a logarithmic* function of the concentration of noradrenaline in the external solution ○ 2 h incubation ● 4 h incubat on

was 0 0018 ml Duplicate samples of these liposome suspensions were incubated with 10 M NA at temperatures from 0° C to 37° C for 2 to 4 h Increasing the tempera ture slightly increased the accumulation of NA into the liposomes

Corresponding experiments were done incubating liposomes for 2 h with $10^{5}$ M 5HT These showed only a minimal increase in accumulation with temperature However even at 37° C the accumulation of 5HT will only be 75 % complete after 2 h judging from the plateau region of Fig 3

*Effect of the concentration of NA on its accumulation by liposomes*

Fig 5 a shows the NA/Rb volume ratios for the accumulation of NA by liposomes at different NA concentrations The incubations lasted 2 h or 4h $^{86}$Rb volume in the 2 h expt was 1 1 $\mu l$ $\mu mol$ $P^{1}$ and in the 4 h expts 1 1 and 1 5 $\mu l$ $\mu mol$ $P^{1}$ The concentration of the radioactive $^{3}H$ NA isotope was kept constant and the carrier NA concentration was varied The figure shows that at higher concentrations of NA the ability of liposomes to concentrate NA from solution goes down suggesting that the liposomes may saturate above $10^{3}$ M NA

Fig 5 b shows the same experimental results as Fig 5 a but calculated to show the molecules of NA bound per molecule of phospholipid at different amine concentra tions The accumulation is not saturated at $10^{3}$ M NA and at this concentration only 1 % of the phospholipid molecules had a noradrenaline molecule attached

TABLE II Influence of liposome composition on the binding of noradrenaline and 5 hydroxytryptamine

| Molar composition of liposomes* | Rb volume ml/mole lipid phosphorus | NA/Rb volume ratio** | $10^4$ × moles NA per mole lipid phosphorus | 5HT/Rb volume ratio * | 10 × moles 5HT per mole lipid phosphorus |
|---|---|---|---|---|---|
| Cholesterol 29 %<br>PC 71 | 1130 | 30 | 6 5 | 11 | 2 3 |
| Cholesterol 29 % ***<br>PC 37 8<br>PE 20 5<br>PS 11 5<br>PA 1 2 | 1050 | 35 | 7 1 | 16 | 3 2 |
| Cholesterol 29 %<br>PC 37 8 %<br>PA 33 2 % | 3300 | 9 | 5 3 | 11 | 6 6 |

* The abbreviations are similar to those in Table I
** Volume ratios were measured for 5 × 10⁻ M NA and 1 63 × 10 M 5HT after 12 h incubation The results were calculated for 2 × 10⁻ M monoamines
*** Standard mixture

*Effect of the phospholipid composition of the liposomes on the accumulation of NA and 5HT by liposomes*

The amount of monoamine absorbed per mole of lipid phosphorus can be calculated from the relation Concentration of monoamine in moles ml⁻¹ × volume (ml) trapped inside liposomes per mole of lipid phosphorus × $(\frac{\text{monoamine}}{\text{Rb}}$ volume ratio $-1)$

Table II shows results obtained for noradrenaline and 5HT after 12 h incubation The concentrations of monoamine actually used to obtain the volume ratio were $5\times10^{-5}$ M (NA) and $1\cdot63\times10^{-5}$ M (5HT) but the results were calculated for $2\times10$ M monoamine to facilitate comparison

*Effect of calcium and osmotic shock on the release of Rb and amines from liposomes*

In the first experiment (Fig 6) liposomes loaded with $^{86}$Rb were prepared in the usual manner After dialysis 2 ml containing 5 9 μmol lipid P ml⁻¹ were incubated for 2 65 h at 37° C with $5\times10^{-5}$ M $^3$H NA They were passed over the Sephadex column diluted and put into 6 tubes so that each tube had 1 5 ml containing the same amount of liposomes in concentrations of CaCl ranging from 0 to 10 mM One ml of each sample was put into a 8/32 Visking dialysis tubing bag and incubated for 1 4 h at 37° C in a shaking waterbath Samples of the liposomes remaining in the bag and the liquid outside the bag were counted for $^3$H and $^{86}$Rb (so that the proportions of the isotopes escaping from the liposome could be calculated) The mean initial volume ratio was 11 4 and the volume $^{86}$Rb trapped per μmol lipid P

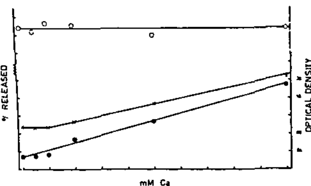



Fig 6 The effect of Ca on the release of Rb and noradrenaline from liposomes at 37 C ● Rb O NA × optical density (O O ) of liposome suspension

was 0 00085 ml The percentage of Rb and NA released as a function of Ca concentration showed that Ca" had no effect whatever on the release of NA but did increase the Rb loss from 1 to 9 % at 10 mM The optical density of the suspension was measured at 300 nm it will be noticed that calcium causes some coagulation of the liposomes as shown by the optical density increase

A second experiment on the same liposome preparation gave a similar result as regards $Ca^{2}$ but showed also that these small vesicles were remarkably insensitive to osmotic shock The liposomes were incubated with 2 5×10 M NA for 3 5 h then put over a Sephadex column After dilution duplicate 1 ml samples containing the appropriate ions were incubated in dialysis bags at 37° C in 20 ml KCl buffer and H O or 20 ml KCl buffer+10 mM CaCl and the loss of both isotopes monitored by counting samples at 15 min intervals The loss of Rb was negligibly small in the KCl buffer and from the sample subject to osmotic shock in H O but the sample in 10 mM CaCl showed a small loss of $^{86}$Rb In one hour all samples had lost 33 % of the NA trapped initially (NA/Rb volume ratio 7 5) no significant differences between samples were found

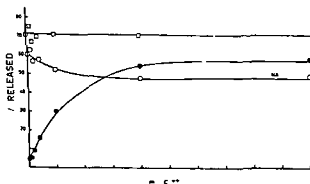



Fig 7 The effect of Ca on the release of Rb and amines from 29 % cholesterol 37 8 PC 33 2 PA liposomes after 1 4 h at 37 C ● Rb O NA □ 5HT

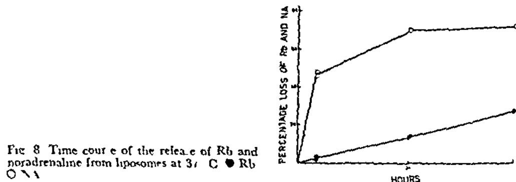



FIG 8 Time course of the release of Rb and noradrenaline from liposomes at 37 C ● Rb ○ NA

An experiment (Fig 7) with more acidic liposomes consisting of 29 % cholesterol 37 8 % PC 33 2 % PA loaded with $7\times10^{-4}$ M $^3$H NA and $2\ 6\times10^{-4}$ M $^{14}$C-5HT together showed results rather similar to the previous experiment The effect of $Ca^{2+}$ on Rb release was even more marked and calcium actually appeared to slightly inhibit the release of NA The initial NA/Rb volume ratio was 18 5HT/Rb ratio was 36 The optical density at 300 nm increased from 0 225 to 0 880

*Release of NA and $^{86}$Rb from liposomes effect of l metaraminol and reserpine*

Fig 8 shows the release of NA and $^{86}$Rb from liposomes made from the standard lipid mixture The lipids were sonicated with $^{86}$Rb and $10^{-4}$ M NA the final liposome suspension contained 2 27 μmol of phospholipids $ml^{-1}$ the $^{86}$Rb volume of this suspension was 0 00223 ml and the volume ratio NA/Rb was 4 87 One ml samples of the suspension were incubated in 8/32 Visking dialysis tubing bags in 10 ml of KCl buffer for 4 24 or 48 h The release of $^{86}$Rb from the liposomes was linear and slow (2 5 % in 4 h and 27 % in 48 h) On the contrary the liposomes lost in 4 h about 50 % of their initial content of NA Thereafter the loss of NA was slower 70 % in 24 h and 73 % in 48 h

The effect of $10^{-5}$ M l metaraminol and $10^{-5}$ M reserpine was studied on the release of NA and Rb during the 4 h incubation Metaraminol did not alter the rate of release of either NA or Rb whereas reserpine decreased the outflow of NA from 47 % to 39 % and increased the outflow of Rb from 2 5 % to 3 6 %

## Discussion

It has been suggested that the membrane phospholipids are important in the storage of catecholamines (for references see Lagercrantz 1971 b) Table I shows the composition of the standard phospholipid mixture used compared to those found in naturally occurring monoamine storage vesicles It will be noticed that in our mixture the neutral lipid sphingomyelin has been replaced by phosphatidyl choline Recently Johnson 1973) showed evidence that cholesterol/phospholipid ratio deter

mines the thickness and surface area (per mol of lipid) of biological membrane. The molar cholesterol/phospholipid ratio chosen in these experiments 0.41 is close to that for monoamine storage vesicles.

The results obtained show that phospholipid liposomes undoubtedly absorb and bind 5HT and NA but in far smaller amounts than are found in storage vesicles. It will be seen from Table II that the binding of NA is virtually unaffected by the presence of acidic lipids but that the binding of 5HT increases. In this context it is instructive to note the pK values for the two amines (Dawson *et al* 1969)

| | | | |
|---|---|---|---|
| NA | 8.82 (OH) | 5HT | 9.8 ($NH_2$) |
| | 9.98 ($NH_2$) | | 11.1 (OH) |

At pH 7.3 both should be substantially unionized. However when a high concentration of acidic phospholipid is present the local pH at the surface of the liposome rises due to the presence of hydroxyl ions in the double layer. If the surface pH was about 3 units higher the NA would have completely ionized acquiring a positive and a negative charge and the 5HT would have only acquired a positive charge and could therefore bind additionally by electrostatic attraction.

The Rb volume per mol of lipid phosphorus depends to some extent on how well the liposomes were sonicated but also on the presence of acidic lipids as shown in Table II. The high value obtained for liposomes with 33 % PA is partly due to the liposomes being larger but also to the presence of excess counter ions as described in Johnson (1973). The 22 ± 6 nm diameter of the liposomes prepared from the lipid mixture shown in Fig 2 is rather smaller than the minimum 26 ± 1 nm diameter that could be expected from previous measurements on aqueous suspensions of liposomes of similar cholesterol/phospholipid ratio (Johnson 1973) or from the relatively large volume trapped per μmol of phospholipid and it is likely that a drying and staining artifact is involved.

It can be calculated that 47 % of the total liposome volume is membrane when the standard lipid mixture is used. This high proportion of membrane is probably the cause of the relative osmotic insensitivity of the liposomes (see Johnson and Buttress 1973) and would explain why the small nerve storage vesicles are less sensitive to hypotonicity than the larger chromaffin granules of the adrenal medulla (Euler and Lishajko 1961 a, Stjarne 1964).

The highest tissue/medium ratios reached for monoamines in liposomes were far below the ones occurring in biological monoamine storage particles (Table III). The accumulation of monoamines into adrenal storage vesicles seems to have a limited specificity for catecholamines since 5HT is taken up even more avidly (Carlsson, Hillarp and Waldeck 1963). In our experiments the initial accumulation of NA by liposomes was 5 times faster than the accumulation of 5HT. This difference in the rate of accumulation is at least partially due to the higher lipid solubility of NA; it was found to be 20 times more soluble in heptane than 5HT at pH 7.3 (37° C). However the final amount of 5HT accumulated may be higher than NA particular

TABLE III Comparison of the uptake storag and realease of noradrenaline and 5 hydroxytryptamine by liposomes and biological storage vesicles

| Phenomenon | Liposomes | Storage vesicles | References for storage vesicles |
|---|---|---|---|
| Highest tissue/medium ratio | 59 (NA)<br>22 (5HT) | 10000 (NA A) (concentration of catecholamines in adrenal granules 0 55 M) | calculated from Carlsson Hillarp and Waldeck (1963) and from Hillarp (1959) |
| Saturation | Not saturated at external NA concentration of 10⁻ M | Saturated at 5 × 10⁻⁴ M NA (adrenal medullary storage granule) | Carlsson *et al* (1963) |
| Rate of adsorption | NA t ½ (plateau) ~ 3 min<br>5HT t ½ (plateau) ~ 15 min<br>Then slow adsorption of both amines | | |
| Rate of release | NA t ½ 4 h<br>(33 % released in 1 4 h) | NA t ½ from adrenal medullary storage granules 1 5 h NA t ½ from nerve trunk storage vesicles ~ 10 min | Stjarne (1964)<br>Euler and Lishajko (1961a) |
| Temperature | At low temperatures slight decrease in uptake of NA and very little effect on uptake of 5HT | At low temperatures marked decrease of uptake | Stjarne (1964)<br>Carlsson *et al* (1963)<br>Da Prada and Pletscher (1968) |
| Osmotic stress | Resistant to osmotic shock | Stores high concentrations of amines (+ nucleotides) against osmotic gradient | Blaschko and Welch (1953)<br>Hillarp Lagersted and Nilsson (1953)<br>Euler and Hillarp (1956)<br>Solatunturi and Paasonen (1966) |
| | | NA easily released from adrenal medullary storage granules nerve trunk vesicles more resistant to osmotic shock | Euler and Lishajko (1961 a)<br>Stjärne (1964) |
| Effect of Ca⁺ | Coagulates vesicles increases rate of Rb loss amine release not affected | No effect on adrenal medullary storage granules Presence of $Ca^{++}$ essential for amine release from cells | Lishajko (1971)<br>Douglas (1968) |
| Effect of reserpine | Decrease of outflow of NA increase in outflow of Rb | Release of stored amine inhibition of spontaneously occurring outflow | Euler and Lishajko (1961 b)<br>Stjärne (1964) |

ly in the case of acidic liposomes When 33 % PA liposomes were prepared in the presence of both 5HT and NA the 5HT/Rb volume ratio was 3 times that of NA Fig 5 a, b show that the NA absorption by the phospholipid did not saturate at an

external NA concentration of $10^{-3}$ M When the amine concentration was $10^{-3}$ M only one phospholipid in 100 had absorbed a molcule of NA The adrenal medullary catecholamine storage particles saturate at $5 \times 10^{-4}$ M (Carlsson Hillarp and Waldeck 1963) These findings suggest that at most the binding to the phospholipid may represent an initial stage in the formation of the monoamine binding complex

The liposomes lost the accumulated NA relatively rapidly 33 % in 1 h and 50 % in 4 h The adrenal medullary granules release their catecholamines at about the same speed whereas the half life of NA release from isolated nerve storage vesicles is less than 10 min (Euler and Lishajko 1961 a Stjärne 1964)

It has often been suggested that the temperature sensitivity of the uptake of monoamines by storage granules and monoamine storing cells is partly due to changes in the lipid structure of the membranes The present experiments show that this hypothesis is untenable as the accumulation of the monoamines by the phospholipid is only very marginally influenced by temperature (Table III and Fig 4)

The site of action of reserpine in the natural granules is either on the storage complex or on the vesicle membrane (for references see Lagercrantz 1971 b) The present experiments showed that reserpine ($10^{-5}$ M) retarded the release of NA from the liposomes this agrees with the effect of reserpine ($10^{-7}$—$10^{-5}$ M) in retarding the spontaneous release of NA from nerve storage granules (Euler and Lishajko 1961 b Stjärne 1964)

Calcium has very little effect on the release of monoamines from liposomes and Lishajko (1971) states that it has no effect on adrenal medullary storage vesicles at physiological concentrations of KCl in the absence of phosphate ions However calcium coagulated the liposomes and it is possible that a similar mechanism is necessary for the binding of a storage vesicle to the membrane where it releases its contents This could explain why no release occurs from cells under biological conditions without calcium present (Douglas 1968)

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Acta physiol scand 1974 90 107—112

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# Effects of 6 OH-Dopamine on the Autonomic Nerves of the Rabbit Myometrium

By

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## Abstract

HERVONEN A and L KANERVA *Effects of 6 OH Dopamine on the autonomic nerves of the rabbit myometrium* Acta physiol scand 1974 *90* 107—112

The degeneration of the terminal network of sympathetic nerve fibres in the rabbit uterus and oviduct were studied after treatment with 6 OH Dopamine The majority of the fluorescent fibres of the myometrial smooth muscle disappeared 24 h after the treatment The recovery of the fluorescence required 4—7 weeks At the finestructural level no adrenergic axon terminals could be identified while the nonadrenergic axons and axon terminals seemed intact. Complete adrenergic denervation can be achieved by the use of 6 OH-dopamine also in those regions which are innervated through the short adrenergic neurones

Evidence for fine structural damage in adrenergic nerves following the use of 6 OH DA (6 hydroxydopamine or 2 4 5 trihydroxyphenalanine) was first reported by Thoenen and Tranzer (1968) and soon the same authors described the effective chemical sympathectomy following the administration of this drug (Thoenen and Tranzer 1968) Since these studies the unique possibilities provided by the 6 OH DA technique have been widely used for studying the distribution of noradrenergic nerves in sympathetically innervated tissue (for ref see Tranzer and Richards 1971)

It is known from the previous studies on the fine structure of the autonomic nerves of different parts of the organism that the autonomic nerves usually contain both adrenergic and cholinergic or non adrenergic axons (for ref see Sporrong 1970) This basic constellation was demonstrated also in the rabbit myometrium (Hervonen and Kanerva 1972 a b 1973)

Chemical sympathectomy with 6 OH DA can be used to produce a situation where only the cholinergic (non adrenergic) component of the autonomic innervation remains functional Therefore more light can be shed on the problems of the functional importance of the autonomic nerves of the female genitals by causing the degeneration of the adrenergic system and continuing studies under such experimental conditions

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Fig 3 Larger magnification of the same field as in Fig 1 Note the internal granular substructure of the inclusions K collagen A axon with neurotubuli F fibroblast M smooth muscle of the uterus ×62 000

bundles of smooth muscle cells can be observed The view is in complete agreement with the earlier report of Owman and Sjoberg (1966)

In all the regions examined (oviduct uteri corpus uteri) a marked reduction in the degree and number of fluorescing nerves when compared with control preparations was obvious 24 h after the injection of 6 OH dopamine The perivascular nerve plexus showed only very weak fluorescence while in the stromal preterminal and terminal network of fluorescent fibres only the finest fibres disappeared completely Some coarser nerves appeared neraly unchanged as far as the intensity of the formaldehyde induced fluorescence is concerned

Three weeks after the treatment a complete loss of FIF of the finer fibres persisted but some coarser ones were easily identified by their FIF which however was weaker than 24 h after the injection A complete recovery of the FIF occurred 4—7 weeks after the injection of 6 OH dopamine The stromal network of fluorescent terminal fibres (short adrenergic neurons Owman and Sjostand 1965 Swedin 1971) seemed to reappear earlier than the perivascular nerves (long adrenergic neurons)

*Electron microscopy* Typical clumps of electron dense material could be noticed in the axons 24 h after injection (Fig 1 2 3 and 5) The inclusions are probably *remnants of adrenergic axons while the nerve endings containing* agranular synaptic vesicles and a few large granular vesicles typical for cholinergic axons seemed to be unaffected After KMnO fixation no adrenergic axon profile could be identified because of the complete lack of all types of small granular vesicles The electron *dense inclusions found within the cytoplasm of the* Schwann cells often displayed a granular substructure (Fig 3) The non adrenergic terminals were not affected by 6 OH DA In spite of the several differences between the short adrenergic neurons and the ordinary long adrenergic neurons (for ref see Swedin 1971) the *degenerative changes produced by large single dosis of* 6 OH dopamine seem to be identical (Malmfors and Sachs 1968 Tranzer and Thoenen 1968 Tranzer and Richards 1971 Hokfelt Jonsson and Sachs 1972)

The autonomic innervation of the rabbit genital tract consists mainly of bundles of preterminal axons which are giving branches to the smooth muscle cells (Hervonen and Kanerva 1972 a 1973) The complete absence of histochemically demonstrable catecholamines as well as the adrenergic synapses indicates that the effect of 6 OH DA is not limited only to the nerve terminals but also to the preterminal more proximal part of the axon The paracervical ganglion through which the genitals are innervated did not show any significant fluorescence histochemical changes after 6 OH DA in the rat (Kanerva and Hervonen to be published Bennett *et al* (1973) studied the innervation of the oviduct of the chick and noticed a marked loss of fluorescence from these structures Instead no real degenerative changes were noticed in the nonterminal axon bundles In the present study also the largest bundles of axons running outside the circular muscle layer of the oviduct showed clear degenerative changes and contained no axons which could have been identified as adrenergic ($KMnO_4$ fixation) As discussed by Bennett (1971) the differences between the reactions of different parts of a neuron to 6 OH DA are probably due to the inherent metabolic differences in these regions The present observations indicate that the functionally terminal axon might be relatively long in the myometrium of the rabbit

From the point of view of further studies on the physiological importance of the autonomic innervation of the rabbit genitals the present study serves as an ultra structural control study The complete lack of adrenergic innervation of the oviduct and the myometrium three weeks after the treatment with 6 OH DA provides possibilities to follow the events of fertilization proceeding of pregnancy possible disturbances of ovulation etc of the denervated animals under ideal conditions

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Acta physiol scand 1974 90 113—123

From the Department of Pharmacology University of Lund Sweden

# Effects of Lanthanum on the Coupling between Membrane Excitation and Contraction of Isolated Frog Muscle Fibres

By

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## Abstract

ANDERSSON K E and K A P EDMAN *Effects of lanthanum on the coupling between membrane excitation and contraction of isolated frog muscle fibres* Acta physiol scand 1974 *90* 113—123

The effects of lanthanum on the electrical and mechanical activities of isolated muscle fibres from the frog were investigated. Lanthanum in the range 0 05—0.3 mM produced characteristic concentration dependent changes of the isometric twitch There was an increase in latent period increase in rate of tension development potentiation of peak twitch amplitude and a prolongation of the relaxation period Lanthanum produced no change of the resting potential but caused a graded reduction in rate of rise amplitude and rate of fall of the action potential At concentrations of the metal ion exceeding 0 3 mM the mechanical response was complicated by impaired excitation leading to a diminution of the twitch response and failure to produce a fused tetanus Exposure to still higher concentrations of lanthanum ($\geqslant$ 0 5 mM) made the fibre electrically inexcitable The lanthanum induced changes were completely reversible on removal of the metal ion from the bath Lanthanum in a concentration of 0 1 mM could be used as a substitute for calcium to maintain a normal resting membrane potential and twitch response The results suggest that lanthanum and calcium act on a common site in the membrane The alterations in twitch kinetics produced by lanthanum are discussed in relation to changes of action potential and mechanical threshold

The essential role of calcium in the excitation and contraction processes in skeletal muscle has been well documented There is evidence that calcium is required in the mechanisms governing the resting and action membrane potentials of the muscle fibre (Ishiko and Sato 1957 Edman and Grieve 1961 1964 Jenden and Reger 1963) Furthermore calcium seems to exert a direct control on the activity of the contractile machinery (for reviews see Sandow 1965 1970 Ebashi and Endo 1968) It has been demonstrated (Hagiwara and Takahashi 1967) that lanthanum binds much more strongly than calcium to the membrane of barnacle muscle fibres and that lanthanum has effects on membrane excitability in lobster axons similar to those produced by calcium in a twenty times higher concentration (Takata *et al* 1966) Lanthanum has also been shown to displace bound calcium from the membrane and to inhibit the transmembrane movements of calcium in smooth muscle (van Breemen 1969

Weiss and Goodman 1969, Goodman and Weiss 1971, van Breemen *et al* 1972) myocardium (Sanborn and Langer 1970 Langer and Frank 1972) and skeletal muscle (Weiss 1970) Furthermore there is evidence in support of the view that lanthanum does not penetrate the cell membrane to any significant degree (Lesseps 1967 Langer and Frank 1972) and therefore that the effects on muscle contractility produced by lanthanum are mainly attributable to an action on the fibre membrane (van Breemen 1969 Weiss and Goodman 1969 Weiss 1970) On this basis lanthanum would seem to provide an interesting tool in the study of the calcium dependent steps in the excitation contraction coupling

The present study is an attempt to elucidate the actions of lanthanum on the time course of twitch and tetanus responses of isolated muscle fibres of the frog These effects were correlated with changes in the action potential and with alterations in the mechanical threshold (Andersson and Edman 1973) The results support the view that lanthanum can replace calcium for the maintenance of a normal resting membrane potential and excitability in a muscle fibre The contractile changes produced by lanthanum can be attributed to effects on membrane functions ordinarily governed by calcium

## Methods

*Preparation* Single muscle fibres were isolated from the ventral head of the semitendinosus muscle of *Rana temporaria* The fibres had a non circular cross-section with the smallest and largest diameters averaging 75 μm and 125 μm respectively Loops of stainless steel wire (weight approximately 0 3 mg) were attached to the tendons as described previously (Edman and Kiessling 1971)

*Muscle fibre chamber* The fibre was mounted in a Lucite chamber between an RCA 5734 mechano-electric transducer and a stainless steel hook the position of which could be set by means of a micrometer screw The chamber was 6 mm wide and 5 mm deep and contained 1 2 ml solution Solutions were introduced at the transducer end of the chamber and were removed by means of a suction drain at the other end In experiments where twitch and tetanus responses were studied the bathing solution was exchanged at approximately 10 min intervals

*Temperature control* The temperature of the bathing fluid was controlled by circulating a water ethylene glycol mixture from a Calora Ultra thermostat through a jacket surrounding the chamber The solutions were stored in jacketed temperature controlled glass containers that were connected through polyethylene tubings (approximately 4 cm long) with a stopcock at the chamber inlet The bath temperature varied between 6 5 C and 7 5 C between different experiments During a given experiment the temperature did not vary by more than ±0 1 C even during exchange of solution

*Determination of sarcomere length* The sarcomere length of the fibre at rest was determined by light microscopy using the approach described previously (Edman 1966) The overall fibre length was adjusted to give a sarcomere length of 2 3—2 5 μm in the different experiments

*Electrical stimulation* The fibre was stimulated by means of a multielectrode assembly as previously described (Edman and Kiessling 1971) Pulses of 1 ms duration were used and the stimulation strength was adjusted to be supramaximal for each electrode pair For tetanic stimulation a 1 s train of pulses with a frequency of 30 to 40 Hz was used After mounting the fibre was tetanized at 10 min intervals during 1—2 h before the actual experiment was started

*Tension recording* Tension was recorded by means of an RCA 5734 mechano-electric transducer The signal was displayed on a Tektronix 502 A oscilloscope and was recorded simultaneously on paper by means of an Elema Schonander ink jet recorder The oscilloscope traces of the twitch and tetanus responses were photographed on 35 mm film

*Recording of membrane potential* Membrane potentials were recorded intracellularly by means of conventional micro-electrodes filled with 3 M KCl Electrodes with a tip potential less than 5 mV and a resistance of 15—25 MΩ were used The reference electrode was an Ag AgCl electrode connected to the bath through an agar Ringer bridge The micro-electrode

and the reference electrode were connected via a high input impedance preamplifier to a Tektronix 502 A oscilloscope The signals were photographed on 35 mm film The amplified signal was also used to modulate an audio frequency generator A successful micro-electrode insertion was indicated by an abrupt change in frequency When action potential recordings were made the fibre was stimulated at one locus only approximately 2—3 mm from the site of impalement

The rate of rise and the rate of fall of the action potential were recorded by electrical differentiation For this purpose the amplifier output was fed through an RC-circuit (time constant 0 1 ms) and displayed on the oscilloscope screen

*Solutions* A Ringer solution of the following composition was used (mM) NaCl 115.5 KCl 2 0 $CaCl_2$ 1 8 *Tris* (hydroxymethyl) aminomethane 2 0 The pH was adjusted to 7 0 by addition of $H_2SO_4$ to a final concentration of 0 94 mM All chemicals used for the Ringer solution were of analytical grade (E Merck Darmstadt) $LaCl_3$ (BDH Chemical Ltd England) was dissolved in the Ringer solution to obtain final concentrations of 0 001—1 0 mM The calcium impurity of the lanthanum chloride did not exceed 0 015 per cent according to the producer s specification Fresh solutions were made up for each experiment.

The water used for washing of glassware and for preparation of solutions was double distilled in borosilicate glass All glass vessels were rinsed before use with 6 N HCl immediately followed by distilled water The muscle fibre chamber was washed with 1 M EDTA solution followed by several rinses with distilled water and finally with Ringer solution

*Measurements of oscilloscope records* Numerical data from recordings of resting membrane and action potentials and isometric tension given under Results refer to measurements from original film records carried out at 20 × magnification in a Nikon comparator

The statistical significance of the results has been calculated using Student s t test

## Results

### I *Effects on twitch and tetanus*

The effects of lanthanum in concentrations ranging between 0 001 mM and 0 03 mM were tested on twitch and tetanus responses of isolated muscle fibres The fibre was paced to produce an isometric twitch at 1 min intervals until the twitch amplitude was completely stable (after approximately 10 min) The bathing solution was then exchanged for a fresh solution of the same temperature containing lanthanum There was no observable change in the twitch myogram in response to 0 001—0 01 mM lanthanum In concentrations between 0 05 mM and 0 3 mM lanthanum produced a graded increase in twitch amplitude Typical effects on the time course of the isometric twitch are illustrated in Fig 1 and from another fibre at a higher sweep rate in Fig 2 It can be seen that lanthanum prolonged the latent period In the experiment illustrated in Fig 2 the latent period increased from 10 1 ms recorded

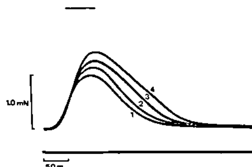



Fig 1 Effects of lanthanum on the isometric twitch response of a single muscle fibre 1 Control normal Ringer 2 0 05 mM lanthanum. 3 0 07 mM lanthanum 4 0 3 mM lanthanum Horizontal line tetanic tension

TABLE I. Effects on resting and action membrane potentials of lanthanum added to calcium containing Ringer solution (Mean ± S.E.). Exposure to lanthanum 10–15 min

| | Number of bundles | Total number of fibres impaled | Resting potential mV | Action potential | | | |
|---|---|---|---|---|---|---|---|
| | | | | Amplitude mV | Maximum rate of rise V/s | Maximum rate of fall V/s | Duration at −25 mV level ms |
| 0 La | 15 | 35 | 91.2 ± 0.3 | 130.9 ± 0.6 | 151.1 ± 3.0 | 40.8 ± 1.0 | 3.7 ± 0.04 |
| La 0.05 mM | 5 | 23 | 92.0 ± 0.5 | 125.3 ± 0.7 | 120.5 ± 3.2 | 29.0 ± 0.9 | 4.2 ± 0.09 |
| La 0.1 mM | 5 | 19 | 92.3 ± 0.5 | 120.2 ± [illegible] | 87.7 ± 4.6 | 20.5 ± 0.8 | 5.3 ± 0.19 |
| La 0.3 mM | 5 | 21 | 91.7 ± 0.4 | 106.2 ± 2.2 | 51.9 ± 2.1 | 14.2 ± 0.5 | 6.0 ± 0.30 |

Differences between means of action potential data obtained in the presence and absence of lathanum are statistically significant ($p < 0.001$).

(Fig. 5). Usually 60–90 min immersion in the calcium free solution was required to depolarize the membrane to −45 mV. Addition of 0.5 mM lanthanum at this stage restored the resting potential to its original value. Elimination of calcium from a bath that contained 0.1–1.0 mM lanthanum had no observable effect on the resting membrane potential over a 1.5 h observation period. When 0.1 mM lanthanum was added in place of the calcium a twitch response of about the same amplitude as in normal Ringer was retained during this time. Higher concentrations of lanthanum (0.5–1.0 mM) abolished the twitch response (*cf.* section I).

The effects of lanthanum on the action potential are demonstrated in Fig. 6 which illustrates intracellular recordings from an isolated single fibre. Lanthanum (0.05–0.3 mM) caused a decrease in the rate of rise of the action potential, reduced the overshoot and prolonged the repolarization phase. These effects were developed with

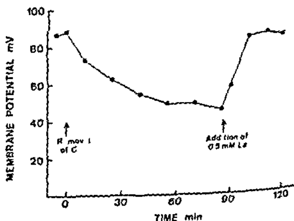



Fig. 5. Decline of resting membrane potential of an isolated muscle fibre immersed in calcium free Ringer solution. Repolarization by addition of lanthanum. The calcium free medium was introduced at zero time (first arrow). Second arrow indicates when the bathing solution was changed to a calcium free Ringer solution containing 0.5 mM lanthanum.

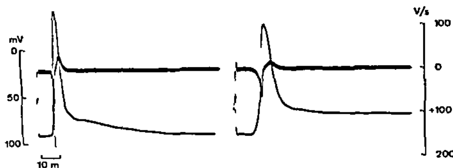



Fig 6 Effects of lanthanum on the action potential of a single muscle fibre Left panel in normal Ringer Right panel in Ringer containing 0 1 mM lanthanum Upper trace dV/dt obtained by electrical differentiation

in approximately 1 min after changing to the lanthanum-containing solution *i e* at the time of the first recording In order to quantify the lanthanum effects on the action potential the single fibre experiments were supplemented with recordings on muscle fibre bundles (containing 3—8 fibres) One or more fibres in a bundle were selected for control recordings in the Ringer solution the remaining fibres being used for recordings in the lanthanum olution The results are given in Table I It can be seen that the action potential changes were graded with the lanthanum concentration At a lanthanum concentration of 0 3 mM the maximum rate of rise was decreased to 1/3 of the control value whereas the duration of the action potential (measured at the −25 mV level) was increased by about 60 per cent With higher lanthanum concentrations the fibre failed to propagate an action potential over its entire length in the presence of 0 5 mM lanthanum the fibres regularly developed a total loss of excitability

Taylor Preiser and Sandow (1972) demonstrated that a slowing of the rise of the action potential was associated with a corresponding delay in the onset of twitch tension In order to test whether the increase in latent period produced by lanthanum (see section I) could be attributed to a later attainment of the mechanical threshold the time was measured from the foot of the action potential (the point at which the trace departed from the base line by 0 2 mV) to the estimated mechanical threshold (−50 mV in normal Ringer and −40 mV in the presence of 0 3 mM lanthanum see Andersson and Edman 1973) This interval was found to increase from 3 2 ± 0 09 ms (mean ± S E n = 15) in normal Ringer to 7 7 ± 0 3 ms (n = 15) in 0 3 mM lanthanum This change is of the same magnitude as the observed prolongation of the latent period (*cf* Fig 2)

## Discussion

*Effects on twitch and tetanus* The present results show that the trivalent cation lanthanum in concentrations of 0 05—0 3 mM causes a graded increase in isometric twitch tension of single frog muscle fibres The lanthanum induced changes of the

isometric twitch were characterized by prolongation of the latent period, increased rate of tension development, potentiation of the twitch amplitude and prolongation of the relaxation period. Other heavy metal ions such as zinc (Edman, Grieve and Nilsson 1966, Sandow and Isaacson 1966, Taylor *et al.* 1972), nickel, cadmium and uranyl (Sandow and Isaacson 1966) also potentiate the twitch in frog skeletal muscle, but they differ from lanthanum in the sense that they do not increase the latent period (Sandow and Preiser 1964, Sandow *et al.* 1965).

The effect of lanthanum upon the latent period is most probably attributable to the fact that lanthanum 1. reduces the rate of rise of the action potential and 2. elevates the mechanical threshold (Andersson and Edman 1973). Both these effects tend to delay the onset of the mechanical activity. It was found (page 119) that the time from the foot of the action potential to the estimated mechanical threshold increased by approximately 5 ms when changing from normal Ringer to 0.3 mM lanthanum. This value compares well with the observed increase in latent period. Zinc, being without effect on the latent period, does not affect the rising phase of the action potential or the mechanical threshold in concentrations (0.002—0.01 mM) that cause a marked potentiation of the isometric twitch in isolated frog muscle fibres (Edman *et al.* 1966).

The twitch potentiation caused by lanthanum, zinc and related metal ions probably involves a common mechanism based on a prolongation of the action potential. As previously demonstrated on the single muscle fibre preparation (Edman *et al.* 1966) and in experiments on whole muscle (Sandow and Preiser 1964, Sandow, Taylor and Preiser 1965, Taylor *et al.* 1972) there appears to be a quantitative relationship between action potential duration (at the −25 mV level) and the duration of the active state. This has been interpreted to mean, as described in detail previously (Sandow *et al.* 1964, Edman *et al.* 1966, Taylor *et al.* 1972), that the action potential governs the time during which activator calcium is released into the myofibrillar space. On this basis, prolongation of the action potential would cause a higher peak concentration of calcium at the contractile sites and this in turn would lead to a delayed elimination of the calcium from the contractile machinery. If the peak concentration of activator calcium were large enough to fully activate the contractile system, an increase in the activator release would merely cause a prolongation of the active state. This would be reflected in the isometric twitch myogram as an increased peak amplitude and a later attainment of the maximum, whereas the initial rate of rise of the twitch would be unaffected. The finding that the speed of tension development was also slightly increased in the present experiments with lanthanum provides evidence that the active state was not merely prolonged but also attained a larger amplitude in response to the elevated myofibrillar calcium concentration. This is consistent with the view that the active state is well below its saturation level during a single twitch in the ordinary Ringer solution at the temperature (7.0° C) used (see Close 1962). It should be added that the rate of isometric tension development would be very little affected by a steepening of the rising phase of the active state. This is evident from the fact (Edman 1970) that the active state

has a very abrupt onset and is almost fully developed within the initial 5—10 ms after the latent period

Lanthanum in concentrations exceeding 0 3 mM depressed the isometric twitch response of isolated frog muscle fibres and this could be accounted for by partial to complete failure of membrane excitation It is reasonable to assume that the same explanation applies to the depressant effect produced by lanthanum at a lower con centration (0 05 mM) in mammalian skeletal muscle (Langer and Frank 1972) At tention has been directed to the finding that lanthanum reduces the movement of calcium across the membrane thta normally occurs during activation of skeletal muscle (Weiss 1970) myocardium (Sanborn and Langer 1970) and smooth muscle (van Breemen 1969) It is unlikely however that lanthanum depresses the mechani cal response in skeletal muscle by virtue of its inhibitory effect upon the transmem brane calcium transport Abundant evidence has been presented indicating that skeletal muscle dos not immediately depend on the extracellular calcium for its ac tivation provided that an adequate resting potential is maintained (Edman and Grieve 1961 1964 Jenden and Reger 1963 Luttgau 1963 Curtis 1963 Armstrong Bezanilla and Horowicz 1972) Further support for this view is provided by the present finding that a muscle fibre retains its ability to twitch for at least 4—5 h in a calcium free medium containing 0 1 mM lanthanum

*Effects on membrane potential* The effects of lanthanum on the resting and ac tion membrane potentials are explainable by postulating that lanthanum similar to certain divalent metal ions (Jenden and Reger 1963) can replace calcium at mem brane sites The pre ent results show that lanthanum in 0 1 mM concentration prevents membrane depolarization after removal of the extracellular calcium Fur thermore addition of lanthanum (0 5—1 mM) produces an almost complete recov ery of the resting potential if the fibre is extensively depolarized in a calcium free medium

Lanthanum also substitutes for calcium in the mechanism underlying the action potential Thus similar to the effects produced by high concentrations of calcium (Ishiko and Sato 1957) addition of lanthanum reduced the amplitude the rate of ri e and the rate of repolarization of the action potential causing an increase in the duration of the action potential Qualitatively imilar effects of lanthanum have been observed in barnacle muscle (Hagiwara and Takahashi 1967) denervated frog muscle (Par ons Johnson and Lambert 1971) lobster axon (Takata *et al* 1966 Blaustein and Goldman 1968 Hafemann 1969) and squid axon (Moore *et al* 1966) It is reasonable to assume that calcium governs the sodium and potassium con ductances by its binding to strategic sites in the membrane and that excitation i based on a momentary dislocation of this calcium (Frankenhaeuser and Hodgkin 1957) As pointed out by Lettvin *et al* (1964) lanthanum having approximately th ame hydrated radius as calcium (3 1 and 2 8 A respectively) would bind mor strongly to the membrane sites than calcium it elf because of its higher valence In accordance with this view it has been shown that much lower concentration c lanthanum than of calcium (concentration ratio approximately 1 10—1 100) ar

isometric twitch were characterized by prolongation of the latent period, increased rate of tension development, potentiation of the twitch amplitude, and prolongation of the relaxation period. Other heavy metal ions such as zinc (Edman, Grieve and Nilsson 1966, Sandow and Isaacson 1966, Taylor *et al.* 1972), nickel, cadmium and uranyl (Sandow and Isaacson 1966) also potentiate the twitch in frog skeletal muscle but they differ from lanthanum in the sense that they do not increase the latent period (Sandow and Preiser 1964, Sandow *et al.* 1965).

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Acta physiol scand 1974 90 124—131
From the Department of Pharmacology University of Lund Sweden

# Effects of Lanthanum on Potassium Contractures of Isolated Twitch Muscle Fibres of the Frog

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## Abstract

ANDERSSON K E and K A P EDMAN *Effects of lanthanum on potassium contractures of isolated twitch muscle fibres of the frog* Acta physiol scand *1974 90* 124—131

The effects of lanthanum on the time course of potassium induced contractures of isolated muscle fibres from the frog were investigated Two kinds of effects could be distinguished 1 A reduction in the rate of tension development and a decrease in contracture amplitude 2 A prolongation of the tension plateau and a decrease in the rate of relaxation during sustained depolarization The effects under point 1 were obtained at lanthanum concentrations $\geqslant$ 0 5 mM while those under point 2 were clearly visible at lanthanum concentrations as low as 0 01 mM In a calcium free medium containing 0 1 mM lanthanum a muscle fibre produced repeatable contractures to 117 5 mM potassium over many hours The slowing of tension development and the diminished contracture amplitude is explainable on the basis of a reduction in the rate of release of activator calcium in response to a depolarization step The prolongation of the plateau and the slowing of relaxation caused by lanthanum is interpreted as reflecting a reduced rate of inactivation of the calcium release mechanism following depolarization

It is well known that the time course of the potassium contracture of frog skeletal muscle is influenced by the extracellular calcium concentration Thus it has been demonstrated that calcium deprivation shortens the contracture time and increases the rate of relaxation whereas an increase in extracellular calcium concentration has the opposite effects (Luttgau 1963 Pauschinger Lorkovic and Brecht 1964 Milligan 1965 Caputo and Gimenez 1967 Foulks and Perry 1967 Frankenhaeuser and Lannergren 1967 Etzensperger 1970 Caputo 1972 b) The calcium induced effects on the potassium contracture can be interpreted to mean that the extracellular calcium in some way controls the release of activator from the intracellular store and/or influences the rate at which activator calcium is resequestered by the sarcoplasmic reticulum

The present investigation was aimed at further elucidating the calcium dependent mechanism governing the kinetics of the potassium contracture Lanthanum seemed

TABLE I Composition of solutions (mM)

| | Solutions | KCl | K CH$_3$SO$_4$ | Na CH$_3$SO$_4$ | NaCl | CaCl$_2$ | CaSO$_4$ | Sucrose |
|---|---|---|---|---|---|---|---|---|
| I | *Normal Ringer* | 2 | — | — | 115.50 | 1.80 | — | — |
| II | Normal Ringer sucrose | 2 | — | — | 115.50 | 1.80 | — | 17 |
| III | Normal Ringer high calcium | 2 | — | — | 115.50 | 1.80 | 8.20 | — |
| IV | 10 K Ringer | 10 | — | 96.90 | 10.60 | 1.80 | — | — |
| V | 16 K-Ringer | — | 16 | 89.96 | 11.54 | 1.80 | — | — |
| VI | 20 K Ringer | — | 20 | 88.99 | 8.51 | 1.80 | — | — |
| VII | 30 K Ringer | — | 30 | 83.03 | 4.47 | 1.80 | — | — |
| VIII | 40 K Ringer | — | 40 | 75.05 | 2.45 | 1.80 | — | — |
| IX | 80 K Ringer | — | 80 | 37.80 | — | 1.50 | — | — |
| X | 117.5 K Ringer | — | 117.5 | 0.77 | — | 1.03 | — | — |
| XI | 117.5 K Ringer sucrose | — | 117.5 | 0.77 | — | 1.03 | — | 17 |
| XII | 117.5 K Ringer high calcium | — | 117.5 | 0.77 | — | 1.03 | 8.97 | — |

All solutions contained 2 mM *Tris*(hydroxymethyl) aminomethane. The pH was adjusted to 7.0 by addition of $H_2SO_4$ to a final concentration of 0.94 mM.

to provide a useful means for this study as this metal ion has been shown to replace calcium in the membrane function (see Andersson and Edman 1973) and probably does not penetrate the cell membrane to any significant degreee (Lesseps 1967, Langer and Frank 1972, van Breemen *et al.* 1972). The actions of lanthanum on potassium induced contractures of single muscle fibres were studied. The results can be taken as support for the view that the effects of lanthanum on contracture development and relaxation are largely attributable to actions on a calcium dependent mechanism in the cell membrane which governs the kinetics of release of activator within the fibre.

## Methods

Single fibres from the semitendinosus muscle of *R. temporaria* were used. The apparatus and techniques employed for mounting the fibres and for recording isometric tension and membrane potential have been described in the preceding paper (Andersson and Edman 1973). A relatively rapid exchange of solution was achieved by flushing precooled solution through the muscle chamber (volume 1.2 ml). The flow rate was 3.0–3.5 ml/s and a flush time of about 2 s was used when the potassium solutions were added for initiation of contracture. This procedure provided a virtually complete exchange of solution as indicated by the finding that the membrane potentials recorded after changing to various potassium solutions (using a 2 s flush) were almost identical to those recorded after a much longer flush time. The temperature varied between 6.5 °C and 7.5 °C in different experiments but was maintained with an accuracy of ±0.1 °C throughout any particular experiment including the temperature fluctuation during exchange of solutions. The bathing fluid was replaced at approximately 10 min intervals.

The compositions of the normal Ringer solution (I) and the various potassium-Ringer

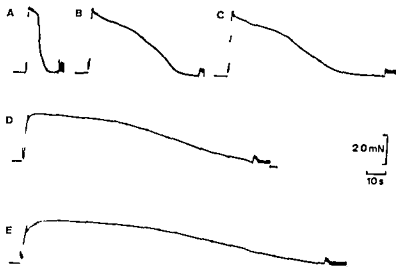



Fig 1 Effects of lanthanum on the time course of the contracture induced by 117 5 mM potassium in a single muscle fibre A Control normal Ringer *B* 0 05 *mM lanthanum* C 0 1 mM lanthanum D 0 5 mM lanthanum E 1 0 mM lanthanum

solutions (IV—X) used for contractures of single fibres are given in Table I The osmolarity and the [K] [Cl] product were made constant by using K and Na methylsulphate All solutions contained 1 8 mM $CaCl_2$ except the 80 mM and 117 5 mM potassium solutions which contained 1 5 mM and 1 03 mM $CaCl_2$ respectively The lower $CaCl_2$ concentrations were used as an attempt to maintain a constant [K] [Cl] product and constant osmolarity It was ascertained in separate experiments that these differences in calcium concentration did not affect the contracture response *Tris(hydroxymethyl) aminomethane (2 mM)* was used as a buffer the pH being adjusted to 7 0 by addition of $H_2SO_4$ to a final concentration of 0 94 mM

Some experiments were performed in which the calcium concentration was increased to 10 mM by addition of $CaSO_4$ to the normal Ringer solution (III) and to the potassium Ringer solution (XII) used for contractures Sucrose 17 mM was added to the control solutions (II and XI) to obtain an equivalent increase in osmolarity

The contracture amplitude was measured to the nearest 0 5 mm from the original paper records the size of the myogram being of the order of 70 mm The highest tension recorded after the flush artifact had subsided was used for the plottings of contracture tension vs membrane potential

## Results

*Effects of lanthanum on the time course of the potassium induced contracture* The effects of lanthanum (0 001—1 0 mM) on the time course of the contracture induced by 117 5 mM potassium were studied The fibre was pre treated with lanthanum for 20—30 min in the ordinary Ringer solution before the 117 5 mM potassium solution (containing the same lanthanum concentration) was applied Between each contracture the fibre was immersed in lanthanum-containing Ringer solution for 20—30 min

Typical results are illustrated in Fig 1 It can be seen that lanthanum prolonged the plateau of the contracture and reduced the rate of relaxation These changes clearly evident at a concentration of 0 01 mM (not illustrated) increased progressively with increasing lanthanum concentrations No clear effects on the rate of rise

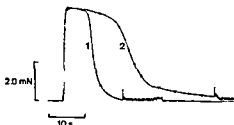



Fig 2 Superimposed records of contractures induced by 117 5 mM potassium in a single muscle fibre 1 Control normal Ringer 2 0 1 mM lanthanum In this fibre there was no pre treatment with lanthanum, the metal ion being applied together with the contracture solution

or maximum amplitude of the contracture were produced by lanthanum in concentrations up to 0 1 mM (Fig 1) At higher concentrations (0 5—1 0 mM) however there was a marked slowing of the tension development and a reduction in peak contracture amplitude

A few experiments were performed in which lanthanum (0 1 mM) was added to the fibre together with the contracture solution *i e* without pre treatment with the metal ion As can be seen from the superimposed records in Fig 2 there was an immediate effect of lanthanum on the contracture time course A comparison with the control record makes evident that lanthanum had already started to act 5 s after it was introduced into the bath *i e* at the point where the control myogram begins to decline However the effect did not become fully developed during this short period of exposure as can be seen by comparing Fig 2 with Fig 1 C

It was shown in the preceding paper (Andersson and Edman 1973) that lanthanum could be used as a substitute for calcium for the maintenance of the resting potential and the twitch response Fig 3 demonstrates that 0 1 mM lanthanum added in place of the calcium also maintained the fibre s contracture ability over a period of at least 4 5 h Furthermore in a fibre that had depolarized in a calcium free medium to

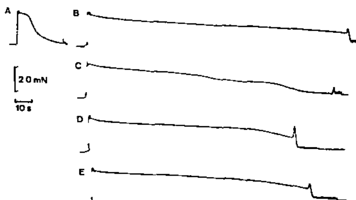
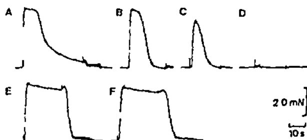



Fig 3 Effects of replacing calcium with 0 1 mM lanthanum on the contracture induced by 117 5 mM potassium in a single fibre A Control normal Ringer B—E After immersion in calcium free lanthanum containing Ringer for 1 5 2 5 3 5 and 4 5 h respectively

Fig 4 Loss of contracture response to 117 5 mM potassium by removal of extracellular calcium and restoration of response by administration of lanthanum Single muscle fibre A Control normal Ringer B—D After immersion in calcium free medium for 30 75 and 105 min respectively Lanthanum 0 1 mM was added to the calcium free medium immediately after record D Records E—F 30 and 75 min respectively after addition of lanthanum These contractures were interrupted before relaxation




an extent that the potassium contracture could no longer be elicited addition of 0 1 mM lanthanum completely restored the contracture amplitude (Fig 4)

If lanthanum acts on a mechanism that is normally governed by calcium changes similar to those produced by lanthanum should be obtained by a mere increase in the extracellular calcium concentration The experiment presented in Fig 5 was performed to test this point Illustrated are contracture responses to 117 5 mM potassium in the presence of 1 mM (trace A) and 10 mM calcium (trace B) As can be seen the increase in calcium concentration caused a reduction in amplitude (to approximately 85 per cent) a prolongation of the plateau and a marked slowing of the relaxation phase i e similar changes as produced by lanthanum

*Effects of lanthanum on the relation between membrane potential and mechanical response* The relation between peak contracture amplitude and potassium concentration was studied in isolated muscle fibres Contracture was induced by rapid exchange of the normal Ringer for solutions of various concentrations of potassium In each fibre a series of measurements with eight different potassium concentrations was carried out first in the ab ence of lanthanum and then in the presence of 0 1 mM lanthanum Between each contracture the fibre was rested in normal Ringer (with or without lanthanum) for 25 min In the second series of contractures (in which lanthanum was included) an intracellular measurement of the resting membrane potential was made immediately after the fibre had relaxed in the respective potassium solution In separate experiments on bundles of muscle fibres (3—8

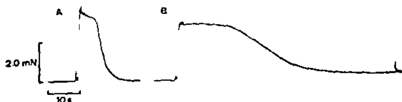



Fig 5 Effects of calcium on the contracture induced by 117 5 mM potassium in a single muscle fibre A Control normal Ringer B 10 0 mM calcium Fibre immersed in Ringer containing 10 0 mM calcium for 20 min before contracture in B

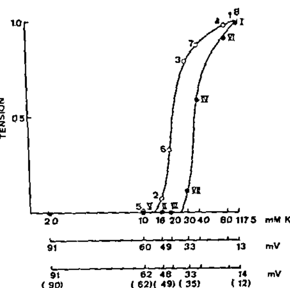



Fig 6 Effect of lanthanum on the relation between peak contracture tension (units) and extracellular potassium concentration (log scale) in a single muscle fibre ○ absence of lanthanum ● presence of 0.1 mM lanthanum Arabic and Roman numerals indicate order that contractures were performed Middle abscissa membrane potentials values recorded in the absence of lanthanum in bundles of muscle fibres Each value represents the mean of 19—40 impalements in at least three different bundles of muscle fibres. Lower abscissa corresponding values in the presence of 0.1 mM lanthanum Values in parentheses refer to single recordings carried out in the presence of lanthanum in the fibre used for the mechanical measurements in this experiment.

fibres) supplementary recordings of the membrane potential were carried out at various external potassium concentrations Fig 6 presents these data and in addition the membrane potential values obtained in a single fibre experiment

Fig 6 shows that the membrane potential at which threshold mechanical activity occurred was close to −50 mV in normal Ringer This value agrees well with results reported in previous studies (Hodgkin and Horowicz 1960 Frankenhaeuser and Lannergren 1967 Anderson 1972) Lanthanum in 0.1 mM concentration shifted the curve relating membrane potential and contracture amplitude to the right increasing the mechanical threshold by 10—15 mV As is evident from the data given on the separate abscissas in Fig 6 0.1 mM lanthanum did not signficantly affect the degree of depolarization at the different potassium concentrations Similar results to those illustrated in Fig 6 were obtained in two other experiments

Attempts were made to investigate the relation between membrane potential and contracture amplitude in the presence of 0.5 mM lanthanum These measurements however were complicated by the fact that lanthanum in this concentration reduced the degree of depolarization in the different potassium media by approximately 10 mV After due allowance for this effect it was found that 0.5 mM lanthanum shifted the mechanical threshold by approximately 15 mV towards less negative potentials

## Discussion

Lanthanum exerts two kinds of effects on the potassium induced contracture of a twitch muscle fibre 1 It reduces the rate of tension development and decreases the contracture amplitude 2 It prolongs the tension plateau and reduces the rate of

relaxation during sustained depolarization of the fibre. These effects are similar to those produced by high concentrations of calcium supporting the view that lanthanum can substitute for calcium at sites that are accessible from the extracellular medium (see also preceding paper Andersson and Edman 1973).

*Amplitude of contracture.* The effects of lanthanum and calcium on the amplitude and rate of rise of the potassium contracture are explainable by assuming that the rate of activator release is reduced at any given depolarization. This would lead to a slower build up of activator-calcium in the myofibrillar space and hence to a slower tension development. The reduced rate of calcium release would also lead to a lower peak concentration of activator at the contractile sites and a decrease in peak contracture amplitude. The effects of lanthanum and high concentrations of calcium could thus be interpreted as meaning that the activator release mechanism has been made less responsive to membrane depolarization. Therefore in order to induce a given rate of activator release a larger depolarization step will be required (*cf.* Fig. 6). By a sufficient increase in the concentrations of lanthanum and calcium in the external medium maximal activator release will not be attained even at full depolarization.

*Time course of relaxation.* The effects of lanthanum and calcium on the duration of the potassium contracture have bearing on the mechanism that determines the transient nature of the contracture response. It is reasonable to assume that relaxation during sustained depolarization is due to a ceasing of the activator release rather than an enhancement of the sequestration function (Hodgkin and Horowicz 1960, Caputo 1972 a and b). The time course of relaxation may thus reflect the rate at which the release mechanism is inactivated after the fibre has been depolarized in a high potassium medium. On this basis the prolongation of the plateau phase of the potassium contracture and the reduced rate of relaxation caused by lanthanum and calcium could be regarded as a slowing of the inactivation of intracellular calcium release during depolarization.

The mechanisms by which lanthanum and calcium 1. reduce the rate of activator release and 2. reduce the rate of inactivation of the release process are still unclear. *It is plausible however that the effects are exerted on a membrane site rather than on a deeper structure.* This accords with the rapid onset of the effect when lanthanum is introduced into the bath. It is of interest to note that mechanisms similar to those proposed above would seem to be valid for the effects of the two metal ions on the sodium conductance in nerve. Thus it has been demonstrated that calcium (Frankenhaeuser and Hodgkin 1957) and lanthanum (Takata *et al.* 1966) not only reduce the magnitude of the initial sodium current in response to a given depolarization step but also decrease the rate at which the sodium carrying system is inactivated. Although the plasma membrane is likely to be a very effective barrier for the trivalent lanthanum ion (Lesseps 1967, Langer and Frank 1972, van Breemen *et al.* 1972), the possibility cannot yet be excluded that enough lanthanum is able to enter the fibre to exert an inhibitory effect upon the sequestration of activator-calcium by the sarcoplasmic reticulum (Batra 1973).

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Acta physiol scand 1974 90 132—142
From the Department of Pharmacology School of Medicine Linkoping Sweden

# Action of Insulin on Vascular and Intestinal Smooth Muscle Effects on Amino Acid Transport, Protein Synthesis and Accumulation of Glucose Carbon

By

HANS J ARNQVIST

*Received 6 March 1973*

**Abstract**

ARNQVIST H J *Action of insulin on vascular and intestinal smooth muscle Effects on amino acid transport protein synthesis and accumulation of glucose carbon Acta physiol scand 1974 90 132—142*

The effects of insulin (0.1 U/ml) on the glucose metabolism in vascular and intestinal smooth muscle are weak in comparison with those in skeletal muscle and also appear later (Arnqvist 1973) In this study insulin (0.1 U/ml) was found to stimulate the membrane transport of α aminoisobutyric acid $^{14}C$ (AIB-$^{14}C$) and the incorporation of leucine $^{14}C$ into protein in bovine mesenteric arteries and rabbit colon smooth muscle Also these effects were weaker and appeared later than the corresponding effects in skeletal muscle The dose respons curves for the accumulation of glucose $^{14}C$ and the incorporation of leucine $^{14}C$ into protein in bovine mesenteric arteries were both displaced to the right in comparison with those for rat diaphragm Blocking protein synthesis by puromycin did not abolish the effect of insulin on the accumulation of glucose $^{14}C$ The sensitivity of bovine mesenteric arteries to the effect of insulin on the accumulation of glucose $^{14}C$ did not increase during the incubation period The finding that all the diverse effects of insulin on smooth muscle were weaker and appeared later than those in skeletal muscle may indicate that they are initiated by a common receptor mechanism which differs in smooth muscle and skeletal muscle

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Insulin (0.1 U/ml) has been reported to stimulate the glucose metabolism in arterial tissue (Wertheimer and Ben Tor 1962 Mulcahy and Winegrad 1962 Lundholm and Mohme Lundholm 1963 Arnqvist 1971 1972 1973) and intestinal smooth muscle (Arnqvist 1973) It has also been found that the effects of insulin (0.1 U/ml) on monosaccharide transport and glycogen synthesis in arterial tissue and intestinal smooth muscle are markedly smaller and appear later than the corresponding effects in rat skeletal muscle (Arnqvist 1972 1973)

In the present investigation the action of insulin on smooth muscle metabolism was further studied The influence of insulin (0.1 U/ml) on the membrane transport of α aminoisobutyric acid $^{14}C$ (AIB-$^{14}C$) and the incorporation of leucine $^{14}C$ into protein was studied to evaluate its effect on amino acid transport and protein syn

TABLE I Effect of insulin (0 1 U/ml) on the distribution of sorbitol [14]C and the total tissue water The tissue samples were incubated for 180 min in a medium containing 0 5 mM unlabelled AIB and 11 1 mM glucose (when present) Sorbitol [14]C was added to make a concentration of 0 5 mM The spaces were calculated as per cent of the wet tissue weight Mean±S E The numbers of observations are given in parentheses

| Preparation | Glucose | Sorbitol space | |
|---|---|---|---|
| | | control | insulin |
| Bovine mesenteric arteries | — | 44 2±1 2 | 44 9±1 1 |
| | + | 44 3±1 7 | 43 4±1 9 |
| Rabbit colon smooth muscle | + | 49 7±1 8 | 5[illegible] 3±2 2 |

thesis The dose response relationship was determined for the effect of insulin on the accumulation of glucose carbon from [14]C labelled glucose and the incorporation of leucine [14]C into protein Since the action of insulin on smooth muscle appeared late its effect on the accumulation of glucose carbon was determined after prior incubation without insulin for varying times to find out if the sensitivity of the tissue to insulin increased during the incubation period The significance of protein synthesis for the effect of insulin on the accumulation of glucose carbon was also examined Bovine mesenteric arteries which have a high content of smooth muscle (Ducret 1930) were used Experiments were also done on an almost pure smooth muscle preparation the muscle layer of rabbit colon

## Materials and Methods

*Animals* Rabbits (2—3 kg) and male rats (60—70 g) of the Sprague-Dawley strain were used Before the experiments the rabbits were starved 20—24 h Fed rats were used Bovine mesenteric arteries were obtained from a slaughter house

*Dissection and incubation* The rabbits were killed by a blow on the neck The part of the colon where the taenia are united and cover one half of the circumference was removed flushed with Krebs-Henseleit buffer at room temperature and cut up longitudinally The mucosal layer was carefully scraped away from the muscle layer Similarly shaped specimens 3—5 mm wide 10—15 mm long and weighing 30—60 mg were prepared from the part covered with taenia Bovine mesenteric arteries were removed 30—60 min after slaughter A 10—15 cm long segment of homogeneous thickness was cut off and transported to the laboratory in Krebs Henseleit bicarbonate buffer at 37 C The buffer was continuously gassed with a mixture of 95 % $O_2$ and 5 % $CO_2$ The arteries were carefully freed from adventitia cut up longitudinally and divided into 4—5 mm broad 8—10 mm long pieces weighing 50—100 mg Histological control showed that almost all adventitia had been removed and no perivascular fat was present The rats were killed by a blow on the neck Cut diaphragms were prepared (Gemmill and Hamman 1941) and divided into hemidiaphragms one part of each pair being used as control and the other as test preparation

The tissue samples were incubated in 25 ml flasks containing 4 ml of medium The flasks were gassed with a mixture of 95 % $O_2$ and 5 % $CO_2$ for 20 s sealed with tightly fitting rubber stoppers and kept in an agitation bath during the preincubation (10—30 min) and incubation periods

*Incubation medium* Krebs-Henseleit bicarbonate buffer with the following composition was used (mM) 120 NaCl 4 7 KCl 1 3 $CaCl_2$ 1 2 $KH_2PO_4$ 25 $NaHCO_3$ The buffer was equilibrated with a mixture of 95 % $O_2$ and 5 % $CO_2$ at 37 C which ga[illegible] 4 1 mg glucose/ml was added to th[illegible]eincubation medium

*Tissue accumulation of labelled substrates* After incubation in labelled substrate the tissue samples were rinsed in buffer for 10 s blotted weighed and dissolved overnight in 1 ml Soluene 9 ml of scintillation fluid (toluene containing 5 g PPO and 0 3 g dimethyl POPOP per liter) was added The isotope content of the tissue was counted in a liquid scintillation

| | Total tissue water | | |
|---|---|---|---|
| effect | control | insulin | effect |
| $0.7 \pm 0.7$ (7) | $77.5 \pm 0.8$ | $76.9 \pm 1.1$ | $-0.6 \pm 0.5$ (8) |
| $-1.1 \pm 1.6$ (6) | $78.1 \pm 0.2$ | $78.0 \pm 0.2$ | $-0.1 \pm 0.2$ (2) |
| $2.6 \pm 2.4$ (5) | $83.1 \pm 0.5$ | $82.9 \pm 0.3$ | $-0.1 \pm 0.2$ (4) |

spectrometer (Packard Tri carb). 100 $\mu$l of the incubation medium was dissolved in 10 ml Instagel and the amount of isotope was measured. Duplicate samples were always counted. The external standard technique was used for quench correction. The counting efficiency was 90—95 %. The tissue accumulation of $\alpha$-aminoisobutyric acid-$^{14}C$ (AIB-$^{14}C$) and sorbitol-$^{14}C$ was calculated as distribution (space) in whole tissue by the following equation

$$\text{distribution } (\%) = \frac{\text{content in wet tissue (cpm/mg)}}{\text{medium concentration (cpm}/\mu\text{l)}} \times 100$$

The tissue content of glucose carbon from $^{14}C$ labelled glucose was calculated as $\mu$mole glucose equivalents by the following formula

$$\frac{\text{cpm/g tissue wet weight}}{\text{cpm/ml incubation medium}} \times \text{glucose concentration } (\mu\text{mol/ml})$$

$= \mu$mol glucose carbon/g tissue wet weight

To determine the distribution of leucine-$^{14}C$ the tissue was homogenized in 10 % trichloroacetic acid (TCA). Duplicate samples of 100 $\mu$l were taken from the protein free tissue extract and the incubation medium and counted for radioactivity. The accumulation of leucine-$^{14}C$ was also expressed as % distribution in whole tissue. Leucine can probably be metabolized in smooth muscle. It is therefore possible that some of the radioactivity in the tissue extract did not represent leucine. This was not analysed in the present study. However under similar conditions more than 90 % of the radioactivity in a protein free tissue extract of skeletal muscle represented the original amino acid (Akedo and Christensen 1962).

*Total tissue water.* After the incubation period the tissue was dried in an oven to constant weight at 100° C. The total tissue water was then calculated as per cent of the wet weight.

*Incorporation of labelled amino acid into protein.* After homogenization in 10 % TCA the precipitated protein was purified exactly as described by Arvill and Ahrén (1967 b). The dried protein was then weighed, dissolved in 1 ml Soluene and counted for radioactivity.

*Chromatography.* Tissue samples were homogenized in 6 % perchloric acid (PCA) and neutralized with $K_2CO_3$. The protein free extract was spotted on precoated cellulose (Avicel) thin layer plates 0.25 mm thick (Analtech Inc.). The plates were developed in butanol : glacial acetic acid : water 120 : 30 : 50. The chromatograms were scanned with an isotope detector (Berthold thin layer scanner).

*Chemicals.* D-glucose-$^{14}C$ (U), sorbitol-$^{14}C$ (U), $\alpha$-aminoisobutyric acid-1-$^{14}C$ and L-leucine-$^{14}C$ (U) were obtained from the Radiochemical Centre, Amersham, England. Instagel and Soluene were commercial preparations of Packard Instrument Company Inc. Monocomponent pork insulin (lot No. MC S 970 AC) was a gift from Novo, Copenhagen.

*Statistical analysis.* Mean values are given ± the standard error of the mean. Adjacent tissue samples from the same animal were used as test and control preparations. The significance of the effect was calculated by Student's t test from the difference between these paired samples. A difference resulting in $p < 0.05$ was considered significant.

## Results

*Effect of insulin on the distribution of $\alpha$-aminoisobutyric acid-$^{14}C$ (AIB-$^{14}C$).* To check that AIB-$^{14}C$ was not metabolized in bovine mesenteric arteries and rabbit

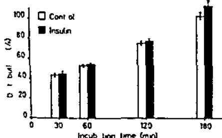



Fig 1a

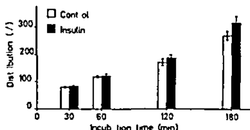



Fig 1b

Fig 1a. Effect of insulin (0 1 U/ml) on the distribution of AIB-$^{14}$C in bovine mesenteric arteries The tissue samples were incubated in 0 5mM AIB $^{14}$C and 11 1mM glucose The asterisks denote the significance of the effect * = < 0 05 ** = p < 0 01 Mean ± S E (n = 10)

Fig 1b Effect of insulin on the distribution of AIB-$^{14}$C in rabbit colon smooth muscle The experimental conditions were the same as in Fig 1 a. Mean ± S E (n = 9)

colon smooth muscle protein free tissue extracts were prepared from tissue samples incubated for 180 min in 0 01 mM AIB $^{14}$C The tissue extracts were then examined by thin layer chromatography It was also investigated if AIB $^{14}$C was incorporated into protein during the incubation period The chromatograms of the tissue extracts showed radioactivity only in the spot corresponding to AIB $^{14}$C and no radioactivity was incorporated into protein This indicates that the model amino acid AIB $^{14}$C is not metabolized in bovine mesenteric arteries or in rabbit colon smooth muscle and could therefore be used to study amino acid transport in these tissues

The distribution of AIB $^{14}$C in bovine mesenteric arteries and rabbit colon smooth muscle was determined after incubation for 30 to 180 min in a medium containing 0 5 mM AIB $^{14}$C 11 1 mM glucose with and without added insulin (0 1 U/ml) Fig 1 a and b show that insulin increased the distribution of AIB $^{14}$C in both tissues The effect appeared after 120 min in bovine mesenteric arteries and after 180 min in rabbit colon smooth muscle Insulin had no effect on the total tissue water or the extracellular space estimated by sorbitol (Table I) It therefore seems probable that the increase in the distribution of AIB $^{14}$C caused by insulin reflected an action on the membrane transport of the amino acid

In bovine mesenteric arteries the effect of insulin on AIB $^{14}$C transport was also studied when no glucose was present in the incubation medium After an incubation time of 180 min the distribution of AIB $^{14}$C (0 5 mM) was 98 8 ± 5 0 % without insulin 105 1 ± 6 3 % with insulin an increase of 6 3 ± 2 6 (p < 0 05 n = 8) No change was found in the total tissue water or the extracellular space estimated by sorbitol (Table I)

AIB transport in skeletal muscle is a complex phenomenon which can be divided into two components (Akedo and Christensen 1962) a) an active transport process that exhibits saturation kinetics and which is saturated at relatively low amino acid

TABLE II. Effect of insulin (0.1 U/ml) on the incorporation of leucine-$^{14}C$ into protein in bovine mesenteric arteries and rabbit colon smooth muscle. The tissue samples were incubated for 180 min in a medium containing 0.01 mM leucine-$^{14}C$ and 11.1 mM glucose (when present). Mean ± S.E. The numbers of observations are given in parentheses.

| Preparation | Glucose | Incorporation of leucine-$^{14}C$ into protein ($\mu$mol/g protein × 180 min) | | |
|---|---|---|---|---|
| | | control | insulin | effect |
| Bovine mesenteric arteries | — | 0.175±0.021 | 0.211±0.023 | 0.035±0.014 (12) $p < 0.05$ |
| | + | 0.175±0.014 | 0.231±0.021 | 0.055±0.012 (12) $p < 0.001$ |
| Rabbit colon smooth muscle | + | 0.332±0.041 | 0.412±0.051 | 0.081±0.026 (15) $p < 0.01$ |

concentrations and b) a first order process similar to passive diffusion and thus not saturable. Similar characteristics were found for AIB transport in smooth muscle when studied in the rabbit detrusor muscle (Osman and Paton 1971). The distribution of AIB-$^{14}C$ (0.5 mM) was 276.6 ± 16.9 % in rabbit colon smooth muscle and 102.4 ± 3.5 % in bovine mesenteric arteries after an incubation time of 180 min. The distribution of AIB-$^{14}C$ therefore exceeded the total tissue water (cf. Table I) in both tissues indicating the existence of active transport systems for AIB. In bovine mesenteric arteries the distribution of AIB-$^{14}C$ was also determined at an AIB concentration of 0.01 mM. At this concentration the distribution of AIB-$^{14}C$ was 129.9 ± 8.5 % (n = 8) after an incubation time of 180 min. The distribution of AIB-$^{14}C$ thus increased when the concentration of AIB in the medium was reduced. This finding suggests that the active transport system for AIB in bovine mesenteric arteries was partially saturated at an AIB concentration of 0.5 mM. When insulin was added the distribution of AIB-$^{14}C$ was increased to 142.1 ± 9.6 % and the increase 12.2 ± 2.4 was significant ($p < 0.01$, n = 8).

*Effect of insulin on the incorporation of leucine-$^{14}C$ into protein.* The incorporation of leucine-$^{14}C$ into protein was determined in bovine mesenteric arteries and rabbit colon smooth muscle after an incubation time of 180 min in a medium containing 0.01 mM leucine-$^{14}C$ and 11.1 mM glucose. In both tissues the incorporation of leucine-$^{14}C$ into protein was moderately increased by the addition of insulin (0.1 U/ml) (Table II). Omission of glucose from the incubation medium did not abolish the effect of insulin (Table II). The time course for the effect of insulin on the incorporation of leucine-$^{14}C$ into protein was studied in bovine mesenteric arteries. From Fig. 2a it is seen that insulin increased the incorporation of leucine-$^{14}C$ into protein in bovine mesenteric arteries. The effect appeared after an incubation time of 120 min and after 180 min the increase produced by insulin was 31.4 ± 6.9 %. For comparison the effect of insulin on the incorporation of leucine-$^{14}C$ into protein in rat diaphragm was determined with the same technique (Fig. 2 b). In rat diaphragm an effect was seen after 30 min, the increase after 180 min was 67.8 ± 15.4 %.

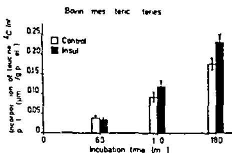



Fig 2a

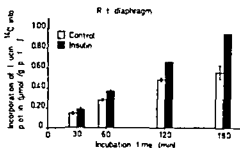



Fig 2b

Fig 2a. Effect of insulin (0 1 U/ml) on the incorporation of leucine $^{14}C$ into protein in bovine mesenteric arteries The tissue samples were incubated in 0 01 mM leucine $^{14}C$ and 11 1 mM glucose The asterisks denote the significance of the effect * = $<$0 05 ** = $<$0 01 *** = $<$0 001 Mean$\pm$S E (n = 12—16)

Fig 2b Effect of insulin on the incorporation of leucine $^{14}C$ into protein in rat diaphragm The experimental conditions were the same as in Fig 2 a. Mean$\pm$S E (n = 5—8)

The distribution of AIB-$^{14}C$ in bovine mesenteric arteries was increased by insulin It was therefore possible that insulin increased the accumulation of $^{14}C$ labelled leucine in the cells and through this effect increased the incorporation of leucine $^{14}C$ into protein To test this hypothesis the effect of insulin on the distribution of leucine $^{14}C$ was determined in bovine mesenteric arteries after incubation for 180 min in a medium containing 0 01 mM leucine $^{14}C$ and 11 1 mM glucose The distribution of leucine $^{14}C$ was 147.3$\pm$9 3 % without insulin and 141 0$\pm$9 3 % with insulin a difference of $-$6 3$\pm$2 5 ($p < 0\ 05$, n = 8) This finding indicates that the observed effect of insulin on protein synthesis was not secondary to a stimulation of the amino acid transport

*Dose response relationship* The incorporation of leucine $^{14}C$ into protein and the accumulation of glucose carbon from $^{14}C$ labelled glucose were studied in bovine mesenteric arteries at varying concentrations of insulin For comparison parallel experiments were performed on the rat diaphragm preparation The incorporation of leucine $^{14}C$ into protein was determined after incubation for 180 min in a medium containing 0 01 mM leucine $^{14}C$ and 11 1 mM glucose In bovine mesenteric arteries the effect of insulin appeared at an insulin concentration of 10 mU/ml (Fig 3) while in rat diaphragm the effect appeared at a concentration of 0 1 mU/ml Compared to rat diaphragm the dose response curve for the effect of insulin on bovine mesenteric arteries was displaced to the right and had a lower maximum

The accumulation of glucose carbon from $^{14}C$ labelled glucose was studied after incubation for 180 min in a medium containing 5 6 mM glucose $^{14}C$ In experiments on bovine mesenteric arteries it was found that the effect of insulin was more easily evaluated when the extracellular glucose $^{14}C$ was washed out by incubating the tissue samples for a further 30 min period in a medium of the same composition but without $^{14}C$ labelled glucose This technique was therefore used to study the effect

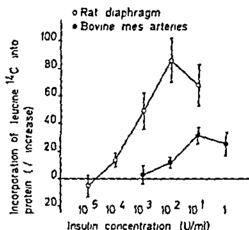



Fig 3

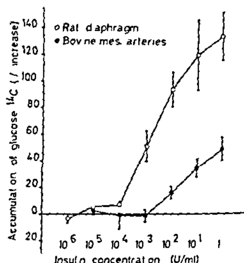



Fig 4

Fig 3 Dose response relationship for the effect of insulin on the incorporation of leucine $^{14}C$ into protein in bovine mesenteric arteries (n = 8—12) and rat diaphragm (n = 6—11) The tissue samples were incubated for 180 min in 0 01 mM leucine $^{14}C$ and 11 1 mM glucose Mean ± S E

Fig 4 Dose response relationship for the effect of insulin on the accumulation of glucose $^{14}C$ in bovine mesenteric arteries (n = 5—9) and rat diaphragm (n = 4—8) All tissue samples were incubated for 180 min in 5 6 mM glucose $^{14}C$ To make the weak effect of insulin on bovine me enteric arteries easier to evaluate the extracellular glucose $^{14}C$ was washed out by incubating the arteries for a further period of 30 min in a medium of the same composition but with unlabelled glucose Values are means ± S E

of insulin on bovine mesenteric arteries Fig 4 shows that the effect of insulin on the accumulation of glucose $^{14}C$ in bovine mesenteric arteries appeared at a concentration of 10 mU/ml while in rat diaphragm an effect was seen at a concentration of 0 1 mU/ml The dose response curve for the accumulation of glucose carbon in bovine mesenteric arteries was displaced to the right in comparison with that of rat diaphragm

*Effect of incubation time on sensitivity of the tissue to insulin* With regard to the slow onset of the action of insulin on smooth muscle it was thought possible that the sensitivity of the tissue to insulin might increase during the incubation period To study this hypothesis bovine mesenteric arteries were incubated without insulin for 10 to 180 min in 5 6 mM glucose The effect of insulin (0 1 U/ml) was then assayed during further incubation for 60 min in 5 6 mM $^{14}C$ labelled glucose Fig 5 shows that the length of the incubation period had no influence on the subsequently determined effect of insulin It therefore seems probable that the sensitivity of the tissue to insulin does not increase with prolonged incubation times

*Influence of puromycin on the effect of insulin* The effect of insulin on the accumulation of glucose carbon from $^{14}C$ labelled glucose was determined after an incubation period of 180 min with and without puromycin in a concentration of 500 μg/ml This puromycin concentration completely blocked the incorporation of

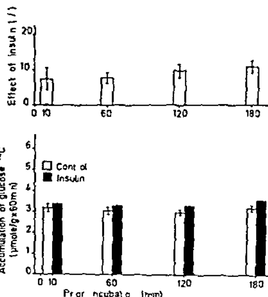



Fig 5 Effect of incubation time on the sensitivity to insulin in bovine mesenteric arteries The tissue samples were incubated for 10 to 180 min in 5 6 mM unlabelled glucose The effect of insulin (0 1 U/ml) was then assayed by determining the tissue accumulation of glucose $^{14}C$ during a further incubation for 60 min in 5 6 mM glucose $^{14}C$ (lower diagram) The upper diagram shows the effect of insulin on the accumulation of glucose $^{14}C$ calculated in per cent of the control value Mean ± S E (n = 8

leucine $^{14}C$ into protein in bovine mesenteric arteries and rabbit colon smooth muscle Thus negligible amounts of radioactivity were found in the protein fractions after addition of puromycin From the results shown in Table III it is clear that the effect of insulin on the accumulation of glucose carbon was not abolished by addition of puromycin to the medium In both tissues the accumulation of glucose carbon was decreased by puromycin

## Discussion

Insulin (0 1 U/ml) moderately increased the distribution of $^{14}C$ labelled $\alpha$ aminoiso butyric acid (AIB $^{14}C$) in bovine mesenteric arteries and rabbit colon smooth muscle The effect of insulin appeared after 120 min in bovine mesenteric arteries and after

TABLE III Effect of puromycin (500 µg/ml) and insulin (0 1 U/ml) on the accumulation of glucose C in bovine mesenteric arteries and rabbit colon smooth muscle The preparations were incubated for 180 min in 5 6 mM glucose C Mean ± S E The numbers of observations are given in parentheses

| Preparation | Puromycin | Accumulation of glucose carbon (µmol/g tissue wet weight × 180 min) | | |
|---|---|---|---|---|
| | | Control | Insulin | Effect |
| Bovine mesenteric arteries | — | 6.99 ± 0.45 | 8.24 ± 0.41 | 1.15 ± 0.31 (12) p 0.01 |
| | + | 4.28 ± 0.32 | 4.76 ± 0.26 | 0.48 ± 0.16 (12) p 0.02 |
| Rabbit colon smooth muscle | — | 7.41 ± 0.38 | 8.49 ± 0.64 | 1.08 ± 0.39 (12) p < 0.02 |
| | + | 5.44 ± 0.17 | 5.96 ± 0.23 | 0.52 ± 0.19 (12) p < 0.02 |

180 min in rabbit colon smooth muscle Insulin had no effect on the extracellular space estimated by sorbitol or the total tissue water The increase in distribution of AIB $^{14}C$ in the tissue was therefore due to an effect of insulin on the membrane transport of the model amino acid Omission of glucose from the incubation medium did not abolish the effect of insulin on the tissue distribution of AIB $^{14}C$ in bovine mesenteric arteries The action of insulin on AIB $^{14}C$ transport was therefore not secondary to an effect of insulin on the glucose metabolism In the diaphragm and the levator ani muscle of the rat the effect of insulin (1 mU/ml) on AIB $^{14}C$ *transport appeared after an incubation time of 15 min and after 120 min the intra* cellular content of AIB $^{14}C$ had increased about threefold (Arvill and Ahren 1967 a) These results indicate that the action of insulin on AIB transport in smooth muscle is weaker and appears later in comparison with that in skeletal muscle

The incorporation of leucine $^{14}C$ into protein was increased by insulin (0 1 U/ml) in bovine mesenteric arteries and rabbit colon smooth muscle after an incubation period of 180 min In bovine mesenteric arteries the effect of insulin on the incorporation of leucine $^{14}C$ into protein was also present when glucose was omitted from the incubation medium showing that this effect was not secondary to a stimulation of the glucose metabolism Since insulin stimulated the membrane transport of amino acid it was thought possible that the effect of insulin on the *incorporation* of leucine $^{14}C$ into protein was due to an increase in the intracellular concentration of leucine $^{14}C$ The distribution of leucine $^{14}C$ in bovine mesenteric arteries was however decreased by insulin It therefore seems probable that insulin besides increasing e amino acid transport also had a direct stimulating action on protein synthesis The effect of insulin on the incorporation of leucine $^{14}C$ into protein appeared after an incubation time of 120 min in bovine mesenteric arteries and after 30 min in rat diaphragm

In this and earlier investigations (Arnqvist 1971 1972 1973) on the action of insulin on bovine mesenteric arteries and rabbit colon smooth muscle insulin was found to increase the monosaccharide transport the glycogen content the incorporation of glucose into glycogen the glycogen synthetase I activity the amino acid transport and the incorporation of amino acid into protein These effects are also produced by insulin in skeletal muscle (Krahl 1961, Narahara and Cori 1968) Insulin thus stimulates the same metabolic processes in both skeletal muscle and vascular and intestinal smooth muscle The effects of insulin on amino acid transport and protein synthesis in skeletal muscle are not secondary to an action on glucose metabolism (Levine 1965) and the effect on protein synthesis is not secondary to the stimulation of amino acid transport (Wool 1965) In this study similar results were obtained for the effects of insulin on amino acid transport and protein synthesis in bovine mesenteric arteries Inhibition of protein synthesis does *not* abolish other effects of insulin on skeletal muscle (Carlin and Hechter 1964) The present results on bovine mesenteric arteries and rabbit colon smooth muscle show that the effect of insulin on the accumulation of glucose carbon in these tisseues was not abolished when the protein synthe was blocked by puromycin Qualitatively the actions of

insulin on smooth and skeletal muscle were therefore similar in several aspects but there were marked quantitative differences

When the effects of insulin (0 1 U/ml) on monosaccharide transport (Arnqvist 1972) glycogen content (Arnqvist 1973) and incorporation of leucine $^{14}C$ into protein were studied using the same experimental technique on bovine mesenteric arteries and rat diaphragm these effects were found to be weaker and appear later in bovine mesenteric arteries than in rat diaphragm The effects of insulin on the incorporation of glucose $^{14}C$ into glycogen and the AIB transport in bovine mesenteric arteries were also less and appeared later compared to those in rat skeletal muscle as discussed in this and a previous report (Arnqvist 1972) In rabbit colon smooth muscle the effects of insulin on monosaccharide transport (Arnqvist 1971) incorporation of glucose $^{14}C$ into glycogen (Arnqvist 1973) and AIB-transport were similar to those in bovine mesenteric arteries Thus the diverse effects of insulin which have been studied in smooth muscle are weaker and appear later in comparison with the corresponding effects in skeletal muscle

Compared to rat diaphragm the dose response curves for the effects of insulin on the accumulation of glucose $^{14}C$ and the incorporation of leucine $^{14}C$ into protein in bovine mesenteric arteries were displaced to the right indicating a lower sensitivity to insulin in bovine mesenteric arteries

A possible explanation for the late appearance of the action of insulin on smooth muscle may be that the sensitivity of the tissue to insulin increases during the incubation time However prior incubation without insulin for up to 180 min had no influence on the effect of insulin on the accumulation of glucose carbon in bovine mesenteric arteries This finding indicates that the sensitivity of the tissue to insulin does not change during the incubation period

It has been suggested that insulin acts on a specific receptor located at the cell membrane and that the interaction of insulin with the receptor initiates a set of signals which in turn evokes the different effects of insulin (Levine 1965 Hechter 1965) Evidence now exists to support this hypothesis Specific receptors for insulin have recently been demonstrated in fat cells (Cuatrecasas 1971) liver plasma membranes (Freychet *et al* 1971) and human lymphocytes (Gavin *et al* 1972) It has also been shown that the intracellular actions of insulin on lipolysis RNA synthesis and glycogen synthetase activity can be initiated through an action of the hormone on the plasma membrane (Cuatrecasas 1969 Turkington 1970 Blatt and Kim 1971) As discussed above the diverse effects of insulin (0 1 U/ml) on bovine mesenteric arteries and rabbit colon smooth muscle all were weaker and appeared later than the corresponding effects in rat skeletal muscle The dose response curves for the effects of insulin on the accumulation of glucose carbon and the leucine $^{14}C$ incorporation in bovine mesenteric arteries were both displaced to the right in comparison with those for rat skeletal muscle The diverse effects of insulin thus seem to be interrelated a finding which may support the idea that they are initiated by a common receptor mechanism The differences in the action of insulin on smooth and skeletal muscle may be due to differences in this receptor mechanism

I am indebted to Mrs Lena Burlin and Miss Gunnel Niklasson for their excellent technical assistance Financial support was given by Nordisk Insulinfond, the Swedish Diabetes Association and the Swedish Medical Research Council (14X 101) Monocomponent pork insulin was generously supplied by Novo Copenhagen

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Acta physiol scand 90 1974 143—152

From the Department of Medical Pharmacology University of Uppsala Sweden

# Carbonic Anhydrase in the Cornea

By

GUDMAR LÖNNERHOLM

Received 6 March 1973

## Abstract

LÖNNERHOLM G *Carbonic anhydrase in the cornea* Acta physiol scand 1974 90 143—152

Carbonic anhydrase activity in the corneas of adult man human fetus monkey rabbit rat, cow pig and American bullfrog was examined by the histochemical method of Hansson In all species the endothelial cells were intensely and distinctly stained for carbonic anhydrase activity The enzyme inhibitor acetazolamide at $10^{-5}$ M in the incubation medium completely and at $10^{-6}$ M almost completely inhibited the staining There was no staining in other parts of the cornea with the possible exception of a few cells in the frog epithelium. Endothelium epithelium and stroma of cow and rabbit corneas were dissected and homogenized The carbonic anhydrase activity was determined by a changing pH indicator method Only the endothelium showed activity and this was sensitive to inhibition by acetazolamide

It has been considered that active ion transport can be reduced by carbonic anhydrase inhibitors in tissues which lack carbonic anhydrase One finding often cited in support of this is that chloride transport in the cornea of the American bullfrog a tissue reported to contain no carbonic anhydrase can be reduced by the carbonic anhydrase inhibitors methazolamide and sulfanilamide (Kitahara *et al* 1967) If indeed there is no carbonic anhydrase in the cornea this finding would contradict the generally held view (see Maren 1967) that sulfonamide inhibitors of carbonic anhydrase act selectively by inhibiting this enzyme It was therefore of interest to reinvestigate whether or not there is any enzyme in the cornea

The corneal stroma has a well known tendency to imbibe fluid Corneal hydration is thought to be controlled by poorly understood active processes across one or both of the limiting cellular layers (Dohlman 1971) To clarify the detailed distribution of carbonic anhydrase in the cornea seemed to be of interest also in this context since the enzyme is known to be present in many actively transporting epithelia (see Maren 1967)

In the present study the carbonic anhydrase activity in the corneas of the American bullfrog and six mammalian species including man has been studied by histochemical and biochemical techniques Preliminary results have been published (Lonnerholm 1972)

I am indebted to Mrs Lena Burlin and Miss Gunnel Niklasson for their excellent technical assistance Financial support was given by Nordisk Insulinfond the Swedish Diabetes Association and the Swedish Medical Research Council (14X 101) Monocomponent pork insulin was generously supplied by Novo Copenhagen

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Abstract

LÖNNERHOLM G *Carbonic anhydrase in the cornea* Acta physiol scand 1974 90 143—152

Carbonic anhydrase activity in the corneas of adult man human fetus monkey rabbit rat cow pig and American bullfrog was examined by the histochemical method of Hansson In all species the endothelial cells were intensely and distinctly stained for carbonic anhydrase activity The enzyme inhibitor acetazolamide at $10^{-5}$ M in the incubation medium completely and at $10^{-6}$ M almost completely inhibited the staining There was no staining in other parts of the cornea with the possible exception of a few cells in the frog epithelium Endothelium epithelium and stroma of cow and rabbit corneas were dissected and homogenized The carbonic anhydrase activity was determined by a changing pH indicator method Only the endothelium showed activity and this was sensitive to inhibition by acetazolamide

It has been considered that active ion transport can be reduced by carbonic anhydrase inhibitors in tissues which lack carbonic anhydrase One finding often cited in support of this is that chloride transport in the cornea of the American bullfrog a tissue reported to contain no carbonic anhydrase can be reduced by the carbonic anhydrase inhibitor methazolamide and sulfanilamide (Kitahara *et al* 1967) If indeed there is no carbonic anhydrase in the cornea this finding would contradict the generally held view (see Maren 1967) that sulfonamide inhibitors of carbonic anhydrase act selectively by inhibiting this enzyme It was therefore of interest to reinvestigate whether or not there is any enzyme in the cornea

The corneal stroma has a well known tendency to imbibe fluid Corneal hydration is thought to be controlled by poorly understood active processes across one or both of the limiting cellular layers (Dohlman 1971) To clarify the detailed distribution of carbonic anhydrase in the cornea seemed to be of interest also in this context since the enzyme is known to be present in many actively transporting epithelia (see Maren 1967)

In the present study the carbonic anhydrase activity in the corneas of the American bullfrog and six mammalian species including man has been studied by histochemical and biochemical techniques Preliminary results have been published (Lonnerholm 1972)

TABLE I Corneas used for histochemistry + is positive staining — negative staining (−) weak and inconstant staining

| Species | Number of corneas | | Carbonic anhydrase activity in the corneal endothelium | |
|---|---|---|---|---|
| | fixed | unfixed | fixed | unfixed |
| adult man | 3 | 2* | − | |
| human fetus | | 3 | | + |
| monkey | 1 | 4 | − | − |
| rabbit | 7 | 3 | + | (+) |
| rat | 5 | 3 | + | − |
| cow | 5 | 2 | + | (+) |
| pig | 2 | | + | |
| bullfrog | 5 | 6 | − | − |

* these corneas lacked endothelium

## Materials and methods

*Histochemical method*

### Preparation of tissue

Corneas from adult man, human fetus, monkey (*Macaca irus*), albino rabbit, rat (Sprague-Dawley), cow, pig and American bullfrog (*Rana catesbeiana*) were used (see Table I).

*The monkeys and the rats were anesthetized with pentobarbital, the rabbits* with urethane and the *bullfrogs* were decapitated. The whole eyes were removed avoiding contamination of the cornea with blood. Fresh *cow* and *pig* eyes were delivered from the local abbatoir. Some eyes were frozen immediately in isopentane cooled with liquid nitrogen. Others were divided with a razor blade and immersed in fixative for 1—2 h. The fixative was 2.5 % glutaraldehyde in 0.1 M phosphate buffer at pH 7.4, but for the bullfrog eyes 1 part of distilled water was added to 2 parts of buffered glutaraldehyde to ensure isotonicity. The glutaraldehyde was prepared from a 50 % stock solution by a one stage vacuum distillation procedure (Anderson 1967). The cornea was then dissected and immersed in fixative for another 2 h, washed in 0.2 M sucrose in 0.05 M phosphate buffer at pH 7.4 (buffered sucrose) for 1—2 h to remove excess fixative and finally frozen.

*Human* corneas were obtained from three eyes with a melanoma in the posterior part of the eye but without signs of involvement of the anterior part. In two cases the intraocular pressure was measured by tonometry and normal values were found. The age of the patients was 5[illegible], 68 and [illegible]8 years. Immediately after removal the eyes were perfused with buffered [illegible] glutaraldehyde (see above) by needles introduced into the anterior chamber and the surface of the cornea was also exposed to the fixative. After 15 min the cornea was dissected and immersed in fixative for 3—4 h, washed with buffered sucrose and frozen.

Human *corneas* were also obtained from two eyes damaged by perforating trauma. They were frozen within one h after removal without any fixation.

*Fetal human* corneas were obtained from three feti 16, 17 and 2[illegible] weeks old. The whole eyes were removed and immediately frozen within one h after the vaginal or caesarean section.

### Staining procedure

The method was described in detail by Hansson (1967, 1968). Briefly the procedures were as follows.

8 µm thick sections of corneas or whole eyes were cut at −20 [illegible] C.

Fixed sections were collected in a Petri dish containing cold buffered sucrose and were transferred to the incubation medium within 3 min. To prevent the *free floating* sections from disintegrating in the slightly effervescent incubation medium, the non ionic detergent, Tween [illegible]80 was added in a final concentration of 1:100 000 (v/v).

Unfixed sections were thawed on a TH WP Millipore® filter 25 µm thick, pore size 0.45 µm (Millipore Filter Corporation, Bedford, Mass. USA) and stained. These section[illegible] were usually allowed to equilibrate with the incubation medium for 10 min under a $CO_2$ atmosphere. Immediate incubation was also tried, however, in an effort to minimize diffusion artifacts.

Fixed sections as well as unfixed sections on their Millipore® filters were floated on the surface of the freshly prepared incubation medium in a 9 5 cm Petri dish The medium contained $CoSO_4$ $1.75\times10^{-3}$ M $H_2SO_4$ $5.3\times10^{-4}$ M $NaHCO_3$ 0 157 M and $KH_2PO_4$ $11.7\times10^{-3}$ M A compound containing cobalt and phosphorous precipitates at carbonic anhydrase sites and is converted to CoS Thus a black precipitate is formed where enzyme is present The volume of the incubation medium was 57 ml and always included 10 ml 1/15 M $KH_2PO_4$ ($=11.7\times10^{-3}$ M) to ensure maximal sensitivity permitting short incubation times of 2—15 min

Sections with and without counterstaining with hematoxylin and eosin were prepared Fixed sections were dehydrated through graded concentrations of ethanol and xylene and mounted with Canada balsam. Unfixed sections on their Millipore® filters were dehydrated in 95 % ethanol absolute n propanol absolute n propanol and xylene (1 1) xylene and mounted in Canada balsam

*Biochemical method*

The carbonic anhydrase activity in dissected parts of cow and rabbit corneas was measured by the changing pH method of Philpot and Philpot (1936) $CO_2$ is bubbled at constant rate through a reaction vessel containing phenol red A standard amount of carbonate is then added causing the indicator to change color The hydration of $CO_2$ to form $H_2CO_3$ neutralizes the added buffer base and causes the indicator to return to the original (acid) color The assays were performed at 0 2 C. One enzyme unit is defined as the amount required to complete the reaction in a time one half of the uncatalyzd reaction One ml was the largest amount of homogenate which could be used for a single determination and the smallest amount of the enzyme that could be detected was 0 1 enzyme unit This roughly corresponds to an amount of 1 3 and $10\times10^{-11}$ moles of the high and low activity forms respectively of the human erythrocyte isoenzymes

Fresh whole *cow* eyes were obtained from the local abbatoir The eyes were put together into a plastic bag and immersed in ice water They were used within two h Before dissection they were rinsed twice in cold saline and corneas with damage or discoloration were discarded The corneal epithelium was scraped off with a knife and the cornea was then removed leaving a 2 mm broad margin to the limbus The endothelium was then gently scraped off The epithelium the endothelium and the remainder of the cornea (mainly corneal stroma) were weighed and each tissue was homogenized separately in cold distilled water containing $10^{-3}$ M EDTA (sodium salt) to protect the enzyme from inactivation by heavy metal ions A tight fitting Teflon® plunger in a glass tube was used. The homogenates were then assayed within a few h Because the stroma was hard to homogenize it was dispersed in a blender (Polytron® Kinematica GMbH Luzern Switzerland) before homogenization This homogenate as well as supernatant resulting from a centrifugation at 35 000 × *g* for 30 min were used

*Rabbit* eyes were removed from albino rabbits anesthetized with urethane avoiding contamination of the corneal surface with blood The corneas were handled like the cow corneas In one experiment however epithelium and stroma were homogenized together

Generally the homogenates were diluted 1 10 The rabbit endothelium and epithelium required higher dilution to obtain enough volume for the enzyme analysis

Anterior chamber fluid was collected from cow and rabbit rabbit eyes by a needle inserted through the intact cornea The fluid was assayed without any dilution

To exclude contamination of the endothelial homogenates by erythrocytes during the tissue preparation the hemoglobin concentration in these homogenates was determined by a peroxidase method (Bing and Baker 1931) Whole blood samples were also collected from cow and rabbit and the carbonic anhydrase activity was assayed

## Results

*Histochemical method*

The whole *endothelial* cells were intensely and distinctly stained in all species when fixed corneas were used (Fig 1—3) The staining was clearly seen already after incubation times of 2—6 min Endothelium from different parts of the cornea was uniformly stained However the most peripheral parts close to the limbus were not studied

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*Human* corneas were obtained from three eyes with a melanoma in the posterior part of the eye, but without signs of involvement of the anterior part. In two cases the intraocular pressure was measured by tonometry and normal values were found. The age of the patients was 52, 68 and 78 years. Immediately after removal the eyes were perfused with buffered 2.5 % glutaraldehyde (see above) by needles introduced into the anterior chamber and the surface of the cornea was also exposed to the fixative. After 15 min the cornea was dissected and immersed in fixative for 3—4 h, washed with buffered sucrose and frozen.

Human corneas were also obtained from two eyes damaged by perforating trauma. They were frozen within one h after removal without any fixation.

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Fig 4 Cornea from human fetus about 16 weeks old unfixed Stained for carbonic anhydrase incubation time 12 min No counterstain Positively stained endothelium ×90

4

With unfixed corneas different results were obtained with different species (Table I) Corneas from the three human feti (Fig 4) and monkey showed intensely stained endothelium In cow and rabbit corneas a weak staining reaction was sometimes found whereas bullfrog and rat corneas were completely unreactive in spite of well preserved endothelium. This indicates a loss of the enzyme by diffusion The unfixed human corneas were clearly damaged since they lacked endothelium and they were also unstained

The *epithelial* cells were always unreactive in unfixed corneas In fixed tissues single cells occasionally showed positive staining in human cow and bullfrog corneas This staining only developed after incubation times of 12—15 min

In human and cow corneas these epithelial cells were located in the outermost cell layer but in the bullfrog cornea stained cells were seen in the basal cell layer of the epithelium (photographs not shown)

The *other parts* of the cornea were always unstained

*Control experiments* The staining was completely inhibited in fixed and unfixed corneas of all species when $10^{-}$ M acetazolamide (Diamox® American Cyanamid Company Pearl River N Y USA) was included in the incubation medium (Fig 2 c) A lower concentration $10^{-6}$ M gave a much delayed and weakened

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Fig 1 Human cornea fixed Stained for carbonic anhydrase incubation time 15 min 1a no counterstain 1 b counterstained with hematoxylin and eosin Vertical black line to the right is the positively stained endothelium. ×120

Fig 2 Bullfrog cornea, fixed Stained for carbonic anhydrase incubation time 12 min 2 a no counterstain 2 b c d counterstained with hematoxylin and eosin 2 a b normal incubation medium positively stained endothelium 2 c acetazolamide $10^{5}$ M added to the incubation medium all staining inhibited 2 d methazolamide $10^{-5}$ M added to the incubation medium all staining inhibited All sections were cut from the same piece of cornea. ×240

Fig 3 3 a cow cornea, fixed. 3 b rabbit cornea, fixed Stained for carbonic anhydrase incubation time 12 min No counterstain. In both corneas positively stained endothelium is seen t the right ×120

The corneal *epithelium* did not show any clearcut carbonic anhydrase activity in any species The epithelial homogenates of cow and rabbit corneas had no catalytic activity The occasional staining of a few cells in the outermost cell layer of human and cow corneal epithelium was probably caused by contamination with hemolysed erythrocytes Monkey rabbit and rat eyes were removed without any contamination with blood and the epithelium was always unstained

In the bullfrog cornea a few cells in the *basal* part of the epithelium were sometimes weakly stained The staining appeared only after relatively long incubation times close to those where the uncatalyzed reaction is known to produce non specific staining Thus a difference in epithelial carbonic anhydrase between mammals and the bullfrog cannot be excluded with certainty at present

*3 Role of carbonic anhydrase in the cornea*

The isolated cornea of the American bullfrog generates a transcorneal potential difference which is produced mainly by an active transport of Cl from aqueous to tear side (Zadunaisky 1966 Ploth and Hogben 1967) This potential difference is reduced by the carbonic anhydrase inhibitors methazolamide or sulfanilamide but not by the inactive analogue Cl 13,850 (Kitahara *et al* 1967) They found no enzyme activity in corneal homogenates and inferred that Cl transport had been depressed by carbonic anhydrase inhibitors in absence of the enzyme They therefore concluded that Cl transport is not dependent on this enzyme and that another enzyme or system had been inhibited The present findings would indicate that the observed reduction in Cl transport could be due to inhibition of carbonic anhydrase after all

Many Cl transporting cells contain carbonic anhydrase (see Maren 1967) but the role of the enzyme is not well understood The results with sulfonamide inhibitors indicate that the enzyme may interact with Cl transport in several ways (Davies and Roughton 1948 Maetz and Garcia Romeu 1964 Hogben 1967 Maren and Broder 1970 Simon and Thomas 1972)

In the mammalian cornea hydration seems to be controlled by a fluid pump in the endothelium This idea is supported mainly by data from the rabbit cornea (Mishima and Kudo 1967 Maurice 1972) and some data from cat and human corneas (Donne and Dohlman 1970) The endothelial fluid pump is not known in detail However the data of Hodson (1971) Dikstein and Maurice (1972) and Fishbarg (1972) suggest that the active fluid transport across the rabbit corneal endothelium is depressed in the absence of $HCO_3$ These findings indicate a possible link between carbonic anhydrase in the endothelial cells and the active process

The question arises if inhibition of carbonic anhydrase might affect corneal hydration and transparency *in vivo* At least in the rabbit inhibitors like acetazolamide and methazolamide enter the aqueous humour and reach concentrations almost equal to those of free diffusible drug in plasma (Wistrand *et al* 1960) Moreover Wistrand (1959) showed that equal concentrations of acetazolamide build up in the anterior aqueous humour and the cornea after systemic administration to

rabbits No visible corneal effects after systemic administration of carbonic anhydrase inhibitors to man or animals seem to have been reported however Foss (1955) observed that the cornea remained clear also after local instillation of acetazolamide into rabbit eyes

Several explanations for a lack of clearly visible corneal effects are possible Very high concentrations of inhibitor may be necessary if high local concentrations of the enzyme exist and only a minor fraction of uninhibited enzyme suffices for the physiological needs In this context it would be important to know which isoenzyme is present in the endothelium since of the two human isoenzymes HCA B and HCA C the former is more difficult to inhibit by the inhibitors presently in clinical use (see Maren 1967) Even a virtually complete inhibition of carbonic anhydrase would not be expected to cause complete inhibition of active transport in the endothelium in agreement with observations in other epithelia (see Maren 1967) Zadunaisky and Lande (1971) showed that the transparency of the bullfrog cornea diminished only after an increase of 30 % or more of the corneal water content Thus slight to moderate increase of the corneal water content after administration of carbonic anhydrase inhibitors may have escaped discovery Measurements of corneal thickness after administration of these inhibitors have not been performed

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*Addendum* After this paper was submitted Silverman and Gerster have reported to us that they have also found carbonic anhydrase in the rabbit corneal endothelium (Exp Eye Res in press)

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In the superfused guinea pig vas deferens prostaglandin E (1—10 ng/ml) caused a dose dependent and reversible depression of the overflow of $^3$H noradrenaline in response to trans mural stimulation Increasing the calcium concentration in the superfusion medium from 1 8—5 4 mM enhanced the overflow of $^3$H noradrenaline and reversed the inhibitory effect of prostaglandin E Closely similar effects were obtained with prostaglandin $E_1$ In the presence of elevated calcium (5 4 mM) prostaglandin $E_2$ was still inhibitory but the dose-effect curve was significantly shifted to the left making the compound less effective in inhibiting the overflow of $^3$H noradrenaline The results are compatible with and add further weight to the considera tion that prostaglandins of the E series restrict the release of noradrenaline from sympathetic nerves by inhibiting the influx of calcium ions into the neuron

It is a well established fact that the release of noradrenaline (NA) from sympathetic nerves is critically dependent on the presence of calcium ions and that increasing the calcium concentration enhances the overflow of NA from stimulated tissues (Hukovic and Muscholl 1962 Kuriyama 1964 Kirpekar and Misu 1967)

Unrelated to sympathetic neurotransmission a close interrelationship between prostaglandins (PGs) and calcium has been demonstrated in many tissues the effect of the compounds varying inversely with the environmental calcium concentration (Mantegazza 1965 Fassina and Contessa 1967 Emmons *et al* 1967 Fassina *et al* 1969) The same appears to hold true also for sympathetic neurotransmission Thus PGEs inhibit the release of NA in response to nerve stimulation in the cat spleen (Hedqvist 1970 a) Increasing the calcium concentration in the perfusion medium counteracts the inhibition and restores normal output figures (Hedqvist 1970 b) On the other hand the NA releasing effect of tyramine which is an calcium independent process (Burn and Gibbons 1965) is not affected by PGE The PGEs have there

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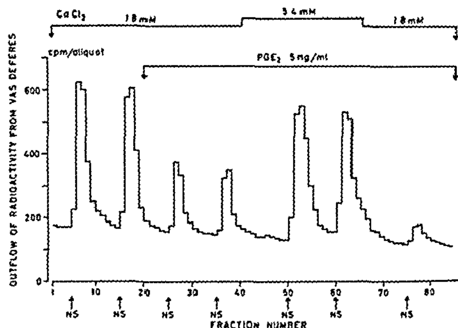



Fig. 1. Guinea pig vas deferens preloaded with $^3H$-NA. Reversal by calcium of inhibitory effect of PGE$_2$ on overflow of $^3H$-NA in response to transmural stimulation (NS) 5 Hz 450 pulses. Time in min = fraction numbers.

fore been postulated to inhibit NA release from sympathetic nerves by restricting the influx into the neuron of Ca necessary for the extrusion of transmitter into the junctional cleft (Hedqvist 1970 a).

The aim of the present study was to determine in the presence of normal and elevated calcium concentration the inhibitory effect of different PGE doses on the overflow of $^3H$-NA from the transmurally stimulated guinea pig vas deferens.

## Methods

The experiments were conducted on male guinea pigs weighing 300—700 g. The animals were killed by a blow on the head and the vasa deferentia were isolated and carefully dissected free from adjacent tissue. The isolated preparation was incubated 1.5 h in Tyrode's solution containing 75 μCi/ml of $^3H$-NA (spec. act. 5.4 Ci/mmol) and was then thoroughly rinsed and continuously superfused in a 2 ml organ bath with NA free Tyrode at a rate of 1 ml/min. The composition of the solution was (concentrations in mM): NaCl 136.7, KCl 2.7, CaCl$_2$ 1.8, MgCl$_2$ 0.5, NaHCO$_3$ 11.9, NaH$_2$PO$_4$ 0.4, glucose 5.5, ascorbic acid 0.1. The solution was kept at 37 °C and bubbled with 5 % CO$_2$ in O$_2$. The preparation was stimulated electrically by means of platinum electrodes in the wall of the bath and a Grass S4 stimulator delivering trains of pulses (5—10 Hz, 1 ms duration, supramaximal voltage) at 10—20 min intervals.

The radioactivity of the different superfusate samples was determined by counting 0.5 ml aliquots in a Packard liquid scintillation spectrometer. As counting medium was used 20 ml of a 3:7 ethanol toluene mixture containing 4 g PPO and 0.1 g POPOP per liter of toluene. Quenching was monitored by internal standards.

and less effective and suggesting that it may act as a reversible competitive in[h]itor of calcium influx into the neuron Theoretically, the PGEs could act directly membrane bound calcium As an alternative assuming that PGEs depolarize the [n]erve terminal membrane (Sjöstrand 1972) reduced influx of calcium may be [sec]ondary to a diminished amplitude of the nerve action potential

[Th]is study was supported by grants from the Swedish Medical Research Council project [N]4342 and from Magnus Bergvalls Stiftelse

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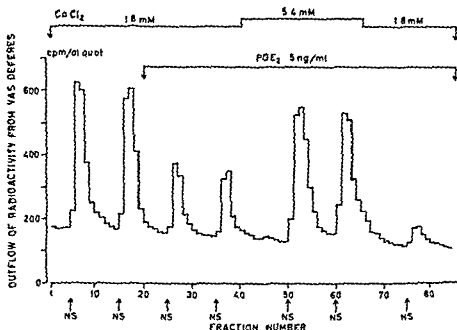



Fig. 1. Guinea pig vas deferens preloaded with $^3H$-NA. Reversal by calcium of inhibitory effect of $PGE_2$ on overflow of $^3H$-NA in response to transmural stimulation (NS) 5 Hz, 450 pulses. Time in min = fraction numbers.

ore been postulated to inhibit NA release from sympathetic nerves by restricting the influx into the neuron of Ca necessary for the extrusion of transmitter into the junctional cleft (Hedqvist 1970 a).

The aim of the present study was to determine in the presence of normal and elevated calcium concentration the inhibitory effect of different PGE doses on the overflow of $^3H$-NA from the transmurally stimulated guinea pig vas deferens.

## Methods

The experiments were conducted on male guinea pigs weighing 500—700 g. The animals were killed by a blow on the head and the vasa deferentia were isolated and carefully dissected free from adjacent tissue. The isolated preparation was incubated 1.5 h in Tyrode's solution containing 25 μCi/ml of $^3H$-l-NA (spec. act. 5.4 Ci/mmol) and was then thoroughly rinsed and continuously superfused in a 2 ml organ bath with NA-free Tyrode at a rate of 1ml/min. The composition of the solution was (concentrations in mM): NaCl 136.7, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.5, $NaHCO_3$ 11.9, $NaH_2PO_4$ 0.4, glucose 5.5, ascorbic acid 0.1. The solution was kept at 37 C and bubbled with 5 % $CO_2$ in $O_2$. The preparation was stimulated electrically by means of platinum electrodes in the wall of the bath and a Grass S4 stimulator delivering trains of pulses (5—10 Hz, 1 ms duration, supramaximal voltage) at 10—20 min intervals.

The radioactivity of the different superfusate samples was determined by counting 0.5 ml aliquots in a Packard liquid scintillation spectrometer. As counting medium was used 10 ml of a 3 : 7 ethanol toluene mixture containing 4 g PPO and 0.1 g POPOP per liter of toluene. Quenching was monitored by internal standards.

pound less effective and suggesting that it may act as a reversible competitive inhibitor of calcium influx into the neuron. Theoretically the PGEs could act directly on membrane bound calcium. As an alternative, assuming that PGEs depolarize the nerve terminal membrane (Sjostrand 1972), reduced influx of calcium may be secondary to a diminished amplitude of the nerve action potential.

This study was supported by grants from the Swedish Medical Research Council (project 04X-4342) and from Magnus Bergvalls Stiftelse.

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Sjostrand N A note on the dual effect of prostaglandin E1 on the motor responses of the guinea pig vas deferens to nerve stimulation *Experientia* (Basel) 1972 *28* 431—432

TABLE I Effect of different PGE doses on H NA overflow from stimulated vasa deferentia in the presence of normal and elevated environmental calcium Mean values±S E from 7 expts The difference between the two sets of data is significant at the 5 % level by Student s t test for paired variates

| H NA overflow % of control stim | | |
|---|---|---|
| PGE ng/ml | CaCl 1 8 mM | $CaCl_2$ 5 4 m |
| 1 | 54±5 | 70±3 |
| 5 | 36±3 | 49±3 |
| 10 | 18±2 | 35±3 |

elevated (5 4 mM) calcium concentration in the superfusion medium As can be seen in Fig 2 PGE (1—10 ng/ml) progressively and reversibly inhibited the over flow of $^3$H NA Subsequent increase of the calcium concentration raised the over flow of $^3$H NA in response to transmural stimulation but PGE was still effective and caused depression of the overflow response Comparing the two ets of data from 7 expts revealed that increasing the calcium concentration in the external medium shifted the dose—effect curve for PGE to the left making the compound less effective in inhibiting $^3$H NA overflow (Table I) The difference between the two sets was significant at the 5 per cent level by Student s t test for paired variates

## *Discussion*

ccording to the available literature the release of NA from sympathetic nerve erminals is critically calcium dependent and it is believed that upon arrival of a ierve action potential depolarization causes an inward movement of membrane $Ca^{++}$ which in turn promotes release of NA into the junctional cleft (Hubbard 1970 Simpson 1968)

Parallelly there seems to exist a close interrelationship between calcium and the action of PGs Thus in many tissues unrelated to sympathetic neurotransmission the action of PGs varies inversely with the environmental calcium concentration (Mantegazza 1965 Fassina and Contessa 1967 Emmons *et al* 1967 Fassina *et al* 1969) *The same appears to hold true also for sympathetic neurotransmission* Thus in the cat spleen increasing the calcium concentration in the perfusion medium counteracts the inhibitory effect of PGE on NA release in response to nerve stimulation On the other hand PGE leaves unaffected the NA releasing effect of tyramine (Hedqvist 1970 b) Therefore the PGEs have been postulated to restrict the release of NA from sympathetic nerves by inhibiting the influx of calcium into the neuron (Hedqvist 1970 a)

The observations in the present study that also in the guinea pig vas deferens increasing the calcium concentration reverses the inhibitory effect of the PGEs on NA overflow add further weight to this consideration Moreover elevating the calcium concentration shifted the dose—effect curve for PGE to the left making the com

NA and after inhibition of local PG formation. Part of the results obtained have been published in a preliminary account (Hedqvist 1973 a).

## Methods

The experiments were conducted on male guinea pigs weighing 500—[illegible]00 g. The animals were killed by a blow on the head and the vasa deferentia were isolated and carefully dissected free from adjacent tissue. The isolated preparation was incubated for 1.5 h in Tyrode's solution containing [illegible] μCi/ml of $^3$H l-NA (spec. act. 5.4 Ci/mMol) and was then thoroughly rinsed and continuously superfused in a 2 ml organ bath with NA free Tyrode at a rate of 1 ml/min. The composition of the solution was (concentrations in mM): NaCl 13[illegible].7, KCl [illegible].7, $CaCl_2$ 1.8, $MgCl_2$ 0.5, $NaHCO_3$ 11.9, $NaH_2PO_4$ 0.4, glucose 5.5, ascorbic acid 0.1. The solution was kept at 3[illegible] °C and bubbled with 5 % $CO_2$ in $O_2$. The preparation was electrically stimulated by means of platinum electrodes in the wall of the bath and a Grass S4 stimulator delivering trains of pulses (5—10 Hz, 1 ms duration, supramaximal voltage) at 10—[illegible]0 min intervals. Contractions of the organ were recorded isotonically.

The radioactivity of the different superfusate samples and organ extracts was determined by counting 0.1—1.0 ml aliquots in a Packard liquid scintillation spectrometer. As counting medium was used 10 ml Instagel or [illegible]0 ml of a 3:7 ethanol-toluene mixture containing 4 g PPO and 0.1 g POPOP per liter of toluene. Quenching was monitored by internal standards.

The gift of the following drugs used is acknowledged: Azapetine (Hoffmann-La Roche, Basel, Switzerland), hydergin (Sandoz, Basel, Switzerland), methoxamine (Burroughs Wellcome, London, England), phenoxybenzamine (Smith, Kline and French, Welwyn Garden City, England), phentolamine (Ciba-Geigy, Basel, Switzerland), prostaglandin $E_2$ (Upjohn Co., Kalamazoo, U.S.A.).

## Results

The NA stores of the guinea pig vas deferens were labelled with $^3$H-NA as described under Methods. Transmural stimulation of the preparation at 5 to 10 Hz consistently caused a marked rise in the outflow of radioactivity, known to consist mainly of intact $^3$H-NA (Hedqvist 1973 b).

*Effect of α receptor blocking agents on $^3$H-NA overflow*

Phenoxybenzamine (PBA, 100 ng/ml) enhanced the overflow of $^3$H-NA in response to nerve stimulation by 265±45 % (mean±S.E., n = 5). This value is somewhat lower but not statistically different from that obtained in a previous study with PBA, 1 μg/ml (Hedqvist 1973 b). The effector response was altered inasmuch the contraction area was markedly depressed while the amplitude remained unchanged or even increased. Closely similar results were obtained both regards to $^3$H-NA overflow and to effector response when hydergine or phentolamine were given in the same dose (Fig. 1).

*Effect of α receptor blockers after inhibition of NA uptake and of PG formation*

After pharmacological blockade of NA uptake and of local PG formation, PBA, hydergine and phentolamine still were effective and enhanced the overflow of $^3$H-NA in response to nerve stimulation (Fig. 2 and 3). As a mean, 100 ng/ml of either PBA, hydergine and phentolamine increased the stimulated overflow of $^3$H-NA by 125±28 % (S.E., n = 11). This effect was completely abolished by a small dose of $PGE_2$ (10 ng/ml).

Acta physiol scand 1974 90 158—165

From the Department of Physiology I Karolinska Institutet Stockholm Sweden

# Role of the α-Receptor in the Control of Noradrenaline Release from Sympathetic Nerves

By

PER HEDQVIST

Received 6 March 1973


## Abstract

HEDQVIST P *Role of the α receptor in the control of noradrenaline release from sympathetic nerves* Acta physiol scand 1974 *90* 158—165

Phenoxybenzamine hydergin and phentolamine all markedly increased the overflow of $^3$H noradrenaline in response to transmural stimulation of the guinea pig vas deferens After pharmacological blockade of neuronal and extraneuronal uptake of noradrenaline and after inhibition of local prostaglandin formation the three compounds were still effective and caused increased overflow of $^3$H noradrenaline This effect was abolished by prostaglandin E Using the same pharmacological blockade noradrenaline and methoxamine inhibited stimulated overflow of $^3$H noradrenaline in a dose dependent manner It is concluded that the activity of α receptors *per se* can feed back regulate the release of noradrenaline from sympathetic nerves The possibility that the receptors are presynaptically located and that they act on Ca influx into the neuron is discussed


It is a wellknown phenomenon that α receptor blocking agents increase transmitter overflow from sympathetically innervated tissues (Brown and Gillespie 1957 and others) The effect of these agents has been attributed to their ability to block the α receptors of the effector cells and to inhibit neuronal uptake of noradrenaline (NA) (Brown and Gillespie 1957 Thoenen *et al* 1964 Brown 1965)

Based on the effect of α receptor blocking agents on transmitter overflow in the cat s skeletal muscle Haggendal (1970) postulated that the reactivity state of the effector cell may in some way feed back control the release of NA from the neuron Similar observations with other organs have supported this view (Farnebo and Malmfors 1971 Enero *et al* 1972 Starke 1972)

Locally formed prostaglandins (PGs) have also been suggested to modulate the release of NA from the neurons (Hedqvist 1970) The aim of the present study was therefore to determine if PGs and α receptors are links in one and the same control system or represent 2 independent means for modulation of NA release from sympathetic nerves The technique used involved determination of the effect of different α receptor blocking and stimulating agents on NA overflow in the guinea pig vas deferens after pharmacological blockade of neuronal and extraneuronal uptake of

NA and after inhibition of local PG formation. Part of the results obtained have been published in a preliminary account (Hedqvist 1973 a).

## Methods

The experiments were conducted on male guinea pigs weighing 500—[illegible]00 g. The animals were killed by a blow on the head and the vasa deferentia were isolated and carefully dissected free from adjacent tissue. The isolated preparation was incubated for 1.5 h in Tyrode's solution containing [illegible]5 µCi/ml of $^3$H-l-NA (spec. act. 5.4 Ci/mMol) and was then thoroughly rinsed and continuously superfused in a 2 ml organ bath with NA free Tyrode at a rate of 1 ml/min. The composition of the solution was (concentrations in mM): NaCl 136.7, KCl [illegible].7, $CaCl_2$ 1.8, $MgCl_2$ 0.5, $NaHCO_3$ 11.9, $NaH_2PO_4$ 0.4, glucose 5.5, ascorbic acid 0.1. The solution was kept at 37 °C and bubbled with 5 % $CO_2$ in $O_2$. The preparation was electrically stimulated by means of platinum electrodes in the wall of the bath and a Grass S4 stimulator delivering trains of pulses (5—10 Hz, 1 ms duration, supramaximal voltage) at 10—20 min interval. Contractions of the organ were recorded isotonically.

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### *Effect of α receptor blocking agents on $^3$H-NA overflow*

Phenoxybenzamine (PBA, 100 ng/ml) enhanced the overflow of $^3$H-NA in response to nerve stimulation by 265 ± 45 % (mean ± S.E., n = 5). This value is somewhat lower but not statistically different from that obtained in a previous study with PBA 1 µg/ml (Hedqvist 1973 b). The effector response was altered inasmuch the contraction area was markedly depressed while the amplitude remained unchanged or even increased. Closely similar results were obtained both regards to $^3$H-NA overflow and to effector response when hydergine or phentolamine were given in the same dose (Fig. 1).

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After pharmacological blockade of NA uptake and of local PG formation, PBA, hydergine and phentolamine still were effective and enhanced the overflow of $^3$H-NA in response to nerve stimulation (Fig. 2 and 3). As a mean, 100 ng either PBA, hydergine and phentolamine increased the stimulated $^3$H-NA by 125 ± 28 % (S.E., n = 11). This effect was completely small dose of PGE (10 ng/ml)

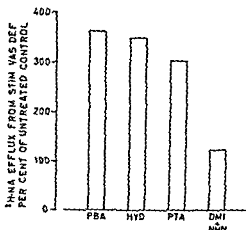



Fig 1 Effect of 100 ng/ml of phenoxy benzamine (PBA) hydergin (Hyd) and phentolamine (PTA) and 400 ng/ml of desmethylimipramine (DMI) + normetanephrine (NMN) on $^3$H NA overflow from stimulated vasa deferentia The columns represent the mean of 3—4 expts PBA Hyd and PTA significant at the 0 1 % level by Student's t test for paired variates DMI + NMN not significant

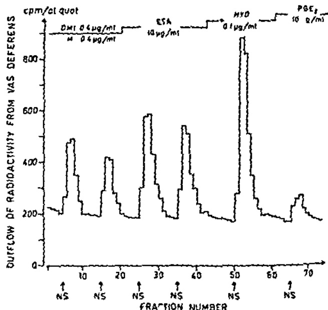



Fig 2 Guinea pig vas deferens preloaded with $^3$H NA Effect of eicosatetraynoic acid (ETA) hydergin (Hyd) and PGE on outflow of tracer in response to transmural stimulation (NS) 450 pulses at 5 Hz Desmethylimipramine (DMI) and normetanephrine (NMN) present throughout the experiment Time in min = fraction numbers

On some occasions the α receptor blocking agent azapetine was used This drug was ineffective in a dose of 100 ng/ml and caused a slight potentiation of $^3$H NA overflow at 1 µg/ml

Blockade of neuronal and extraneuronal uptake was achieved with desmethylimipramine (DMI) and normetanephrine (NMN) 0 4 µg/ml of each When vasa deferentia were incubated with $^3$H NA ($5 \times 10^{-7}$ M) the two drugs caused 80 4 ± 1 1 % (mean ± S E n = 6) inhibition of uptake and retention of $^3$H NA Further addition of PBA 0 1—1 0 µg ml did not alter the inhibition When DMI and NMN were given to the superfused vas deferens they caused a slight increase of the ove

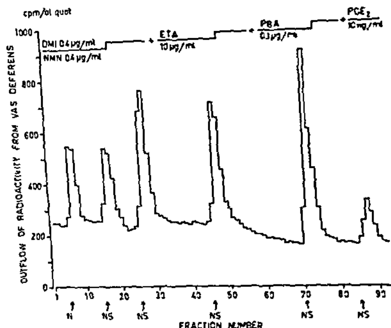



Fig 3 Guinea pig vas deferens preloaded with ³H NA Effect of eicosatetraynoic acid (ETA) phenoxybenzamine (PBA) and PGE₂ on overflow of tracer in response to transmural stimulation (NS) 450 pulses at 5 Hz Desmethylimipramine (DMI) + normetanephrine (NMN) present throughout the experiment Time in min = fraction numbers

flow of ³H NA in response to nerve stimulation (Fig 1) and meanwhile often moderately decreased the effector response

Eicosatetraynoic acid or indomethacin (10 µg/ml) were used to inhibit local PG formation This dose has been shown to block the release of PG and to increase the efflux of NA from the stimulated vas deferens (Hedqvist 1972 [illegible] Hedqvist 1973) (cf Fig 2 and 3)

### *Effect of α receptor stimulating agents on ³H NA overflow*

In the presence of DMI NMN and eicosatetraynoic [illegible] caused inhibition of ³H NA overflow in response to [illegible] inhibition was seen in most cases with 10 ng/ml [illegible] creased with the dose (Fig 4) A NA dose of 10 to 100 [illegible] the mechanical response while 1 µg/ml increased [illegible] contraction of the organ in response to nerve [illegible]

Methoxamine was also tested with respect to [illegible] using the same procedure as with [illegible] depressed the efflux of ³H NA and [illegible] effective in lower concentration [illegible]

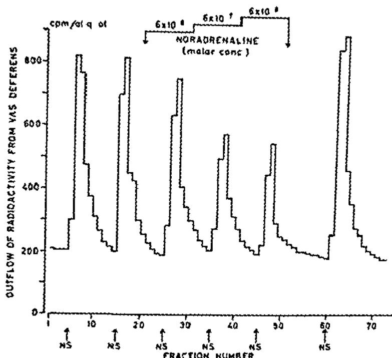



Fig 4 Guinea pig vas deferens preloaded with $^3$H NA Effect of increasing doses of NA on overflow of tracer in response to transmural stimulation (NS) 450 pulses at 5 Hz Desmethyl imipramine + normetanephrine (0 4 $\mu$g/ml nad indomethacin (10 $\mu$g/ml present throughout the experiment Time in min – fraction numbers

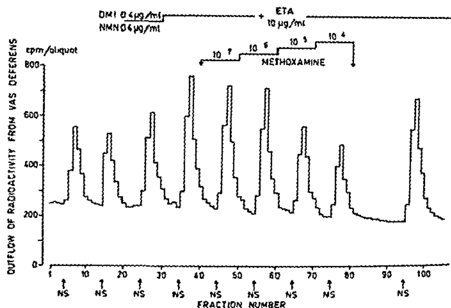



Fig 5 Guinea pig vas deferens preloaded with $^3$H NA Effect of increasing doses of methoxamine on overflow of tracer in response to transmural stimulation (NS) 450 pulses at 5 Hz Desmethylimipramine (DMI) normetanephrine (NMN) and eicosatetraynoic acid (ETA) present to inhibit tissue binding and local prostaglandin formation Time in min = fraction numbers

## Discussion

The technique used in the present study involved transmural stimulation of the guinea pig vas deferens There is good reason to believe that this means specific activation of postganglionic nerve fibres since the stimulation parameters exclude direct stimulation of the effector cells Moreover the effector response is completely abolished by guanethidine and tetrodotoxin while it is left unchanged after hexamethonium (Hedqvist and Euler 1972)

The overflow of NA from the organ was monitored by determining the efflux of ³H NA previously taken up into the nerves (cf Hertting and Axelrod 1961) Almost all of the radioactivity retained in the organ and the majority of that overflowing in response to stimulation was represented by intact ³H NA (Hedqvist 1973 b) Total radioactivity is therefore an appropriate indicator of ³H NA and in all probability also of endogenous NA

Four compounds with α receptor blocking properties (PBA hydergin phentolamine azapetine) were found to increase the efflux of ³H NA from the stimulated vas deferens On the other hand NA and methoxamine both with α receptor stimulant properties caused a decreased overflow of ³H NA Closely similar results using one or more of these or related compounds have been obtained with mouse vas deferens rabbit heart and nictitating membrane (Farnebo and Malmfors 1971 Starke 1972 Enero *et al* 1972)

α Receptor blocking agents are generally believed to increase the overflow of NA by inhibiting its binding to tissues (Brown and Gillespie 1957 Thoenen *et al* 1964) However PBA hydergin and phentolamine were all effective and caused a marked increase of ³H NA efflux after inhibition of neuronal and extraneuronal uptake of NA Moreover DMI and NMN which are potent inhibitors of uptake, and uptake (cf Iversen 1967) did not cause but a small increase of stimulated ³H NA efflux Similarly cocaine which is a potent inhibitor of NA uptake causes a moderate potentiation of NA overflow when compared to PBA (Hughes 1971) Finally PBA significantly increases stimulated efflux of ³H NA from cat nictitating membrane in doses too low to affect neuronal uptake of NA Enero *et al* 1972) Therefore only a small portion of the effect of α receptor blocker can be explained by inhibition of NA uptake and implies that their main action is at the α receptor level

Evaluation of the effect of α receptor blockers on NA overflow is complicated by their possible interference with the formation of PGs In a variety of sympathetically innervated tissues NA release is depressed by small doses of exogenous PGEs and increased after inhibition of local PG formation (Hedqvist 1970 1972 Wennmalm 1971 Chanh *et al* 1972 Fredholm and Hedqvist 1973) Since PBA inhibits the efflux of PG from stimulated tissue (Davies *et al* 1967) part of its effect on NA overflow has been explained in terms of removal of a PG mediated mechanism operated to control NA release from the nerves (Hedqvist 1969 1970)

In order to overcome this difficulty either of two potent inhibitors of PG formation were used The dose given was the same as that previously shown to block the efflux of PGs from the stimulated vas deferens (Hedqvist 1972, Fredholm and

Hedqvist 1973). In the presence of DMI+NMN to inhibit neuronal and extraneuronal uptake and eicosatetraynoic acid or indomethacin to inhibit PG formation PBA, hydergin and phentolamine were still effective and caused increase of $^3$H-NA overflow in response to nerve stimulation. Thus it seems established that α-receptor blockade removes a discrete, not PG mediated, mechanism for control of transmitter release from sympathetic nerves.

If the activity of α-adrenergic receptors controls the release of NA from the nerves by a feed-back mechanism, then α-receptor stimulating agents would be expected to decrease the overflow of NA from stimulated tissue. This was found to be the case since both NA and methoxamine inhibited the efflux of $^3$H-NA from the stimulated vas deferens in the presence of DMI+NMN and eicosatetraynoic acid or indomethacin. Admittedly fairly high concentrations of NA and methoxamine had to be used. However, methoxamine is not taken up by the nerves (Iversen 1967) and the vas deferens is very insensitive to exogenous NA. The reason for this is presumably that the nerve terminals are deeply enbedded in grooves of the effector cells (Lane and Rhodin 1964).

The reactivity state of the effector cells has been postulated to feed-back regulate NA release from the nerves (Häggendal 1970). Considerable support for such a transsynaptic regulatory mechanism derives from the effect of α-receptor inhibiting and stimulating agents on NA overflow in several tissues (Farnebo and Malmfors 1971, Enero *et al.* 1972, Starke 1972).

If the activity of the α-receptors on the effector cells determines the release of NA from the nerve terminals, one has to consider the presence of a hypothetic chemical messenger to overleap the synaptic cleft. Such a messenger can only in part be explained in terms of a PG. Moreover, *e.g.* PBA increases the overflow of transmitter also in tissues in which the proportion of α-receptors in the effector organ is small or absent (Adler *et al.* 1970). Therefore an alternative may be provided by postulating the presence of presynaptic α-receptors on the nerve terminals (Enero *et al.* 1972). As a consequence NA released from the neuron will activate the receptors which in turn restrict further release of NA in response to forthcoming nerve action potentials. Part of the inhibition may be due to active receptors triggering PG formation in the nerve terminal, but a fraction of the inhibition must be an action on the process of excitation-secretion coupling apart from the PG system. Presently the target for the action of the presumed presynaptic receptors cannot be defined. However, since PGs reverse the effect of α-receptor blocking agents on NA overflow, the two systems postulated to feed-back regulate NA release may have a single denominator. It is concievable that both PGs and presynaptic receptors act in one way or the other to inhibit influx of Ca⁺⁺ necessary for the ultimate extrusion of NA into the synaptic cleft.

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# Group I Inhibition in Ib Excited Ventral Spinocerebellar Tract Neurones

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## Abstract

LINDSTROM S and E D SCHOMBURG *Group I inhibition in Ib excited ventral spino cerebellar tract neurones* Acta physiol scand 1974 *90* 166—185

The convergence of inhibition and excitation from muscle spindle Ia and Golgi tendon organ Ib afferents was investigated in ventral spinocerebellar tract (VSCT) neurones monosynaptically excited from Ib afferents Although most cells received Ib excitation from several nerves the dominating excitation was supplied from nerves to synergistic muscles acting as either flexors or extensors at one joint Disynaptic Ia IPSPs from the nerve to the knee extensor were common in cells with their main Ib excitation from the nerves to either hip knee or ankle extensors or to knee flexors The Ia IPSPs in cells excited from hip extensors and knee flexors were depressed by antidromic impulse in motor axons while no such effect occurred on Ia IPSPs in the other cells The Ia IPSPs in Ib VSCT neurones thus seem to be mediated by two different groups of Ia inhibitory interneurones with and without recurrent inhibition from motor axon collaterals through Renshaw cells Disynaptic Ib IPSPs from nerves to thigh muscles were rare while pre sumed Ib IPSPs from nerves to ankle and toe muscles were more common In some cells there were indications of convergence of monosynaptic EPSPs and disynaptic IPSPs from Ib afferents in the same nerve The results are discussed in relation to the hypothesis that the VSCT relays information about interneuronal transmission in the spinal cord

The ventral spinocerebellar tract consists of three main groups of cells two of which receive monosynaptic excitation from either Golgi tendon organ *(Ib) afferents* (Oscarsson 1956 1957 Eccles *et al* 1961) or large muscle spindle (Ia) afferents (Lundberg and Weight 1971) the third group is without monosynaptic excitation from primary afferents (Lundberg and Weight 1971 *cf* also Eccles *et al* 1961 Lundberg and Oscarsson 1962) It has recently been shown that disynaptic Ia IPSPs in the latter two groups of cells can be depressed by impulses in motor axons (Gus tafsson and Lindstrom 1970 1973) just as Ia IPSPs in motoneurones (Hultborn *et al* 1971 a) indicating that the same group of Ia inhibitory interneurones ter minates on both motoneurones and VSCT neurones This finding has been inter preted as supporting the hypothesis (Lundberg 1971) that the VSCT relays informa tion about interneuronal transmission in the spinal cord (Gustafsson and Lindstrom 1973 Lindstrom 1973)

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Disynaptic IPSPs from Ia afferents are evoked also in Ib excited VSCT neurones (Eccles *et al* 1961) In view of Lundberg's VSCT hypothesis it seems important to know whether the Ia IPSPs are recurrently depressed also in these cells However too few cells of this type were found in the earlier study (Gustafsson and Lindström 1973) to obtain an unequivocal result The recurrent effects on Ia IPSPs have now been studied in a larger sample of Ib VSCT neurones The results demonstrate that two types of Ia IPSPs occur in these neurones one being recurrently depressed by impulses in motor axons the other being unaffected The occurrence of one or the other type of Ia IPSPs seems to be related to the pattern of convergence of Ib excitation onto the cells Since the Ib VSCT neurones may signal information about Ib pathways to motoneurones (Lundberg 1971) the convergence of inhibition from Ib afferents onto the neurones was also studied

## Methods

The experiments were done on 10 adult cats operated under ether anesthesia and subsequently anesthetized with α chloralose (i v initially 50—60 mg/kg and additional 10—20 mg/kg after 5—6 h) During the recording the cats were immobilized with gallamine triethiodide (Flaxedil May and Baker Ldt) and artificially respired Four cats were spinalized at the level of Th 12 while in the remaining 6 cats the right spinal half (contralateral to the recording side) was left intact to allow antidromic activation of VSCT neurones from cerebellum (dorsal funiculi and left spinal half transected at Th 12) In the latter animals the left anterior lobe of the cerebellum was exposed Several muscle nerves in the left hindlimb (*cf* Abbreviations) and the ventral roots L5—S1 on the left side were transected and their proximal ends mounted for electrical stimulation Intracellular recordings were obtained from Ib VSCT neurones on the left side of the spinal cord The cells were identified as belonging to the VSCT by their input from ipsilateral muscle nerves and by antidromic invasion either from the contralateral spinal half at Th 12 (Oscarsson 1957 Eccles *et al* 1961) or from the ipsilateral anterior lobe of cerebellum (unipolar cathodal stimulation of the cerebellar surface with rectangular current pulses of 0 2 ms duration and of a strength not exceeding 2 mA Lundberg and Oscarsson 1962 Gustafsson and Lindstrom 1973) The microelectrodes used were filled with 2 M K-citrate solution and had a tip diameter of 1 5—2 0 μm and a resistance of 3—6 MΩ Otherwise the dissection the maintenance of the preparation and the technique of recording and stimulation were as described previously (Gustafsson and Lindstrom 1973)

*Abbreviations* The following abbreviations are used Ventral spinocerebellar tract VSCT ventral root VR excitatory postsynaptic potential EPSP inhibitory postsynaptic potential IPSP flexor reflex afferents FRA anterior biceps and semimembranosus ABSm adductor femoris Add deep peroneus (without cutaneous and extensor digitorum brevis branches) DP flexor digitorum and hallucis longus FDL gastrocnemius and soleus G S gracilis Grac hamstring (PBSt and ABSm together) Ham posterior biceps and semitendinosus PBSt plantaris Pl quadriceps Q sartorius Sart tibial (without calf muscle branches) Tib

## Results

The present report is based on observations from 65 intracellularly recorded VSCT neurones which received monosynaptic excitation from Golgi tendon organ (Ib) afferents Most cells were identified as belonging to the VSCT by antidromic invasion from the contralateral spinal half at low thoracic level but principal findings to be described have been observed also in cells identified as VSCT neurones by antidromic invasion from the ipsilateral anterior lobe of cerebellum (totally 14 cells *cf* Methods) Although the Ib VSCT neurones were searched for in several segments (L3—L6) they were with a few exceptions found in the lower part of the L4 segment and the upper part of the L5 segment confirming the results of Hubbard

and Oscarsson (1962) As judged from microelectrode readings they were usually located more medially in the spinal cord than Ia excited VSCT neurones even if there semed to be a great deal of overlap between the location of these two groups of VSCT neurones (*cf* Hubbard and Oscarsson 1962 Burke *et al* 1971 Jankowska and Lindstrom 1970)

To get the highest possible sample of Ib cells the efforts were concentrated on trying to impale neurones which in extracellular recordings were antidromically fired from the contralateral spinal half and orthodromically fired by a single or a short train of maximal group I volleys in any of the dissected nerves but not by similar volleys in Ia afferents 13 cells with Ib excitation found in an earlier study (Gustafsson and Lindstrom 1973) have also been included Final classification of the cells as Ib VSCT neurones was done after carefully grading the EPSPs by varying the strength of stimulation of the nerves from which they were evoked Usually the double volley technique (Bradley and Eccles 1953 Eccles *et al* 1957 a) was used to differentiate effects evoked from Ia and Ib afferents The EPSPs were considered to be evoked from Ib afferents if they grew with the second components of the incoming nerve volley from nerves displaying a double configuration of the group I volley (usually nerves to thigh muscles) or in the higher threshold group I range from nerves with single contoured group I volleys (Bradley and Eccles 1953 Eccles *et al* 1957 a b)

Examples of typical Ib EPSPs are shown in Fig 1 A—F At a stimulus strength

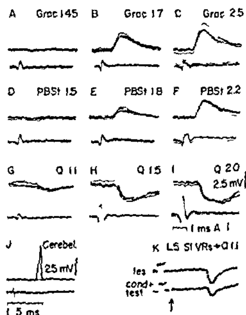



Fig 1 *Convergence of monosynaptic excitation from Ib afferents and disynaptic inhibition from Ia afferent in a VSCT neurone* Upper traces in A—J are intracellular responses lower traces are nerve volleys recorded triphasically from the dorsal root entry zone Positivity is indicated upwards in intracellular recordings and downwards in nerve volley recordings A–C and D–F monosynaptic EPSPs evoked by stimulation of *Ib afferents in the Grac and PBST nerves* respectively G I disynaptic IPSPs from Ia afferents in the Q nerve *The strength of the* nerve stimulation in multiples of the threshold strength is indicated above the records J antidromic spike evoked by surface stimulation of the ipsilateral anterior lobe of cerebellum (Cerebel) K averaged records of submaximal test Ia IPSP from the Q nerve (upper trace) and of test IPSP conditioned by a preceding maximal stimulation of VRs L5—S1 (lower trace Each trace is the average of 20 responses Arrow indicates the arrival of the ventral root volley to the spinal cord The voltage calibration in I refers to intracellular responses in A–I Calibration pulses in K are 1 mV in amplitude and 2 ms in duration (Extracellular field potentials were recorded just outside this and the following cells after withdrawal of the microelectrode but since these field potentials usually were very small compared to the synaptic potentials they are not included in the figures)

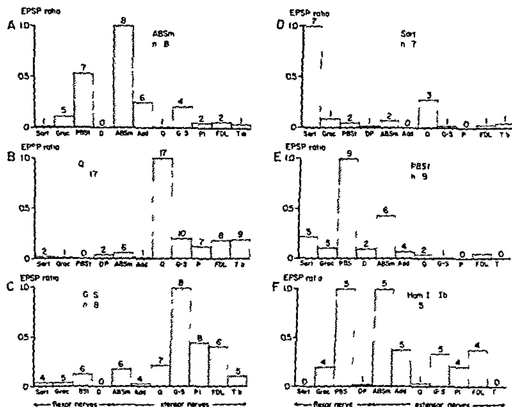



Fig 2 *Convergence of monosynaptic group I EPSPs from different nerves onto Ib VSCT neurones* A—E show the results for groups of cells with the largest Ib EPSPs from the ABSm Q G S Sart and PBSt nerves respectively The histogram in F is for cells with convergence of Ia and Ib excitation from the hamstring nerve (ABSm or PBSt) The histograms give the relative amplitude of the mean EPSPs from each nerve in relation to the amplitude of the mean EPSPs from the nerve supplying the largest EPSPs in the group of cells The figures above each staple indicate the number of cells in the group in which the given nerve evoked a detectable EPSP The mean amplitudes of the largest EPSPs in A—F were 3 7 mV 4 3 mV 9 6 mV 3 1 mV 2 4 mV and 4 5 mV respectively Further details in the text

measured under quite different membrane potential conditions in different cells and the various groups of Ib VSCT neurones are not comparable with respect to the state of the cells For instance the EPSP values for the G S group were obtained mainly from hyperpolarized cells in excellent condition while corresponding values for the PBSt group were obtained mainly from depolarized cells This means that the absolute values of the EPSPs (in mV cf legend of Fig 2) should be considered with great caution The relative effects from the different nerves should be less affected by the cell condition since the EPSPs from the different nerves always were recorded in rapid succession However since the number of cells in each group is small (cf Fig 2) only larger differences are significant as for instance the differences between the effects from the PBSt and Add nerves or Add and Grac nerves in Fig 2 A ($p < 0.05$ Wilcoxon signed rank test Although ratios of mean EPSPs are used in Fig 2 principally similar results are obtained if the means of EPSP ratios from individual cells are calculated

The Ib excited VSCT neurones fell into 5 groups with the largest EPSPs from the nerves to either the hip knee or ankle extensors ABSm Q and G S or to the hip or knee flexors Sart and PBSt Two cells not included in Fig 2 obtained their largest EPSPs from the nerves to the knee flexor Grac and the toe extensor FDL respec

tively These cells had a convergence similar to that in the PBSt and G S cells except that the EPSPs from the Grac and FDL nerves were larger than the EPSPs from the PBSt and G S nerves As apparent from Fig 2 there were usually large differences in amplitudes between the largest EPSP in a cell and EPSPs from the other nerves However a certain degree of overlap between the different groups seems to exist since for instance one cell in the PBSt group received an almost equally large EPSP from the ABSm nerve and the reverse was true for one cell in the ABSm group Similar tendencies were found for single cells with respect to the Q—G S G S—ABSm G S—Pl G S—FDL nerves

The general impression from the histograms in Fig 2 is that Ib VSCT cells with their largest EPSPs from a nerve to an extensor muscle receive convergence of excitation mainly from nerves to other extensors and that cells with their largest EPSPs from a nerve to a flexor muscle are mainly excited from other flexors For instance the Q cells (Fig 2 B) received excitation from the ankle and toe extensors G S Pl FDL Tib and to a lesser extent from the hip extensor ABSm but hardly anything from the flexors The occurrence of large EPSPs from the Q nerve in some cells mainly excited from the nerve to the hip flexor Sart (Fig 2 D) is presumably not in contradiction to this rule In a few experiments the Q nerve was divided into the branches to the pure knee extensor vastocrureus and to the rectus femoris which also has a hip flexor function One of the Sart cells was recorded in such an experiment and in this cell the Q EPSP was supplied exclusively by afferents in the rectus femoris branch It is quite possible that the same was true also for the two other Sart cells with excitation from the Q nerve The major divergences from the suggested pattern are the strong excitation found in ABSm cells from the nerve to the knee flexor PBSt and the reversed situation in PBSt cells However this convergence may rather be an exception since the AB and PB nerve branches innervate two functionally different parts of the same biceps muscle (Sherrington 1910) Even if any middle branch to the muscle (*cf* R M Eccles and Lundberg 1958) is omitted (as was done in the present experiments it is quite likely that the remaining AB and PB nerve branches are not functionally pure with respect to the Ib afferents Particularly it seems possible that the nerve to the AB division of the muscle contains Ib afferents from Golgi tendon organs in the origin part of the PB division of the muscle Even if anatomical factors thus may be responsible for the found convergence of effects from the PBSt and ABSm nerves a functional meaning of this convergence should of course not be excluded since for instance ABSm motoneurones receive a substantial Ia excitation from the PBSt nerve (R M Eccles and Lundberg 1958) In view of the highly differentiated convergence onto other VSCT neurones (Lundberg and Weight 1971 Lindstrom and Schomburg 1973) the occasional occurrence of effects in some cells from unusual nerves (*cf* Fig 2) should not be considered as insignificant since these cells may represent functional subgroups

Eccles *et al* (1961) found one cell which seemed to receive convergence of monosynaptic excitation from Ia and Ib afferents in the same nerve Similar cells

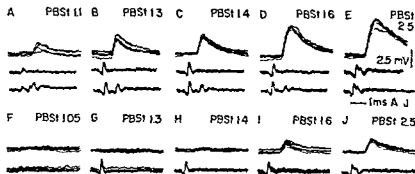



Fig 3 *Convergence of monosynaptic excitation from Ia and Ib afferents in a VSCT neurone* A—E and F—J are from two different cells recorded in the same cat A—E monosynaptic EPSPs evoked from Ia and Ib afferents in the PBSt nerve Lowermost traces in A—E  bins double volley tests The strength of the testing (second) stimulus was supramaximal for group I fibres while the strength of the conditioning stimulus was similar to that used in the above records F—J monosynaptic EPSPs evoked from Ib afferents in the PBSt nerve The voltage calibration in E refers to intracellular responses in A—J Further details as in Fig 1

been found in the present study and the effects from the PBSt nerve onto one of these cells are shown in Fig 3 A—E This cell was recorded in a cat with perfect division of the PBSt nerve volley into Ia and Ib components as revealed by testing with the double volley technique (*cf* lowermost traces in A—E) The Ia component was maximal at a stimulus strength of about 1 25 times threshold and the Ib component started to be recruited at about 1 4 times threshold and was maximally activated at about 2 0 times threshold The threshold for the EPSP in the cell was very low (A) and a maximal Ia volley evoked an EPSP of about half maximal size (B) There was no further increase in the EPSP amplitude when the stimulus strength was increased to 1 4 times threshold (C) but the EPSP started to grow again with the Ib component (D) the maximal amplitude of the EPSP evoked by a slightly supramaximal group I volley is shown in E Although Ib EPSPs may receive a small contribution from the Ia volley due to contamination of the Ia component with Ib fibres this seems to be an unlikely explanation for the large EPSP in Fig 3 especially since the EPSP grew in several steps between A and B and between C and E Further other cells in the same preparation received pure Ib EPSPs from the PBSt nerve (Fig 3 F—J) Totally 6 cells (in 5 different cats) received large EPSPs from afferents in both the Ia and Ib component of nerves with good Ia/Ib separation In 5 of these cells the largest EPSPs were supplied by any of the PBSt or ABSm nerves (hamstring while one cell received its largest EPSP from the G S nerve The convergence of excitation from other nerves onto the hamstring Ia/Ib cells is shown in Fig 2 F in the same way as in A—E In 4 of these cells there was a clear Ia and Ib contribution to the EPSPs from both the RBSm and PBSt nerves while for the other nerves the Ia/Ib constribution to the EPSPs could not be assessed for certain even if the EPSPs in most cases grew through the whole group I range The total group I effects are therefore shown in Fig 2 F That these VSCT cells with convergence of Ia and Ib excitation represent a specific group of VSCT neurones different from

TABLE I *Convergence of disynaptic Ia IPSPs in Ib VSCT neurones* The table shows the number of cells which received the indicated IPSP in relation to the number of tested cells The neurones are classified as in Fig 2 In the G S and PBSt groups there is included one cell excited from the FDL and Grac nerves respectively (cf section I)

| Type of IPSP | Type of VSCT neurone | | | | | |
|---|---|---|---|---|---|---|
| | ABSm | Q | G S | Sart | PBSt | Ham Ia+Ib |
| Q Ia | 8/8 | 8/22 | 9/9 | 0/7 | 11/12 | 5/5 |
| Sart Ia | 1/8 | 1/21 | 0/9 | 0/7 | 1/11 | 0/5 |
| Sart gr I | 3/8 | 3/21 | 1/9 | 0/7 | 1/11 | 2/5 |

VSCT neurones different from VSCT neurones excited from either Ia or Ib afferents is further indicated by the differently organized Ia inhibitory pathway to them (*cf* section II)

## II *Disynaptic Ia IPSPs*

*Convergence of Ia IPSPs* Eccles *et al* (1961) found that many Ib VSCT neurones received disynaptic Ia IPSPs from the Q nerve This was true for cells with their largest Ib EPSP from either the hamstring or the Q nerves Similar observations have been made in the present study Disynaptic Ia IPSPs from the Q nerve were evoked in 41 of the studied 63 cell Examples of such Q Ia IPSPs are shown in Fig 1 G—I and Fig 4 E—F The IPSPs appeared already with very small Ia volleys (Fig 1 G) and there was no further increase in amplitude when the stimulus strength was increased to activate also Ib fibres (Fig 1 H and I) The occurrence of Q Ia IPSPs in relation to the pattern of Ib excitation is shown in Table I where the cells are classified as in Fig 2 Quadriceps Ia IPSPs were evoked in all groups of Ib cells except those with the main Ib excitation from the Sart nerve A considerable proportion of the Q cells also lacked Q Ia IPSPs The segmental latencies of the Ia IPSPs varied between 1 1—1 6 ms (mean 1 4 ms) which means that they were on the average about 0 6 ms longer than the real central latencies of the Ib EPSPs in the cells Disynaptic Ia IPSPs were evoked also from the Sart nerve in 3 cells (Table I) in additional 10 cells there were disynaptic group I IPSPs from the Sart nerve the Ia/Ib nature of which was not asessed Undubious Ia IPSPs were not found from any other nerves in the Ib VSCT neurones

*Recurrent effects on Ia IPSPs* In VSCT neurones excited from Ia afferents and also in some VSCT neurones without monosynaptic excitation disynaptic Ia IPSPs are recurrently depressed by impulses in motor axons (Gustafsson and Lindstrom 1973) In the present study the effects of antidromic impulses in the VRs L5—S1 were tested on Q Ia IPSPs in 37 VSCT neurones A single ventral root volley maximal for α fibres and preceeding the test volley by 7—10 ms was used (Hultborn *et al* 1971 a Gustafsson and Lindstrom 1973) It was soon apparent that the Ia IPSPs were susceptible to recurrent depression only in some of the VSCT neurones

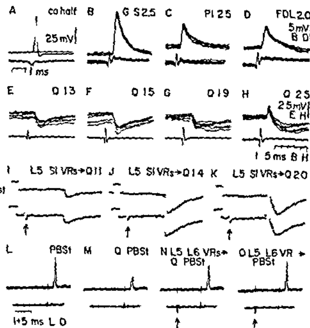



Fig 4 *Lack of effects of a ventral root volley on Ia IPSPs in a Ib VSCT neurone* A antidromic spike evoked by stimulation of the contralateral spinal half (co half) B—D monosynaptic EPSPs evoked by maximal group I volleys in the G S Pl and FDL nerves respectively F—H disynaptic Ia IPSPs and monosynaptic Ib EPSPs from the Q nerve I—K upper traces disynaptic test Ia IPSPs evoked by stimulation of the Q nerve with different strengths lower traces test IPSPs conditioned by a preceding maximal volley in the VRs L5—S1 Each trace is the average of 20 responses I—O monosynaptic reflexes recorded from the VR S1 (upper traces) L a PBSt test reflex M the PBSt reflex inhibited by a submaximal Ia volley in the nerve to the antagonist Q (Q 1 2 × threshold) N decreased effectiveness of the Q Ia inhibition when preceded by a maximal stimulation of the VRs L5—L6 O direct effect on the PBSt test reflex of the L5—L6 ventral root volley Arrows in I—K and N—O indicate the arrival of the ventral root volleys to the spinal cord Calibration pulses in I—K are 1 mV in amplitude and 2 ms in duration Further details as in Fig 1

Of 37 tested cells the Ia IPSPs were clearly depressed in 19 were unaffected in 15 and for 3 cells it was not possible to decide if a small effect was present or not

These findings are exemplified by records in Fig 1 and 4 The cell in Fig 1 received its largest Ib EPSPs from the Gmc (A—C) and PBSt (D—F) nerves The disynaptic Ia IPSP from the Q nerve (G—I) was clearly depressed by a volley in the VRs L5—S1 as shown by the averaged records in K The cell in Fig 4 received the largest EPSPs from the G S Pl and FDL nerves (B—D) The disynaptic Ia IPSP from the Q nerve is shown in E—F When the stimulation of the Q nerve was increased to activate Ib fibres there appeared also a monosynaptic Ib EPSP from the Q nerve (G—H) In this cell there was clearly no effect on the Ia IPSP of an antidromic volley in the VR L5—S1 (I—K) Great care was taken to exclude that small effects were overlooked in cells like the one in Fig 4 The conditioning effects were tested many times (usually more than ten averaging runs) with test IPSPs evoked by different strengths of stimulation of the Q nerve (*cf* I—K) mostly small submaximal IPSPs were tested since the recurrent depression is more effective on such IPSPs (Hultborn *et al* 1971 a)

Cells without recurrent depression of the Q Ia IPSPs were found in six different preparations The lack of effect cannot be explained by nonfunctioning preparations since in most cases VSCT neurones with recurrent effects on their Q Ia IPSPs were

Rec depr

No rec depr

Largest EPSP from
ABSm
PBSt
Grac
Q
G S
FDL
Ham Ia Ib

Fig 5 *Comparison between the susceptibility of Ia IPSPs to recurrent depression and the pattern of Ib excitation of of the VSCT cells* The cells are classified as in Fig 2 and each symbol represents one tested cell For the cell in the "middle group it was not possible to decide if a small effect was present or not

found in the same preparation just before or after the cells without effects (13 cells) Further it was regularly controlled that the used ventral root stimulation was adequate to release PBSt motoneurones from Q Ia inhibition using monosynaptic test reflexes The records in Fig 4 L—O show one such control series taken immediately after the records in I—K The PBSt monosynaptic test reflex (L) was conditioned by a preceding Q Ia volley (M) The Ia inhibition of the PBSt motoneurones was much less effective if preceded by a ventral root volley in the VRs L5—L6 (N) due to recurrent inhibition of the Q Ia inhibitory interneurones The L5—L6 ventral root volley alone had only a small inhibitory effect on the PBSt motoneurones (O) showing that the increase in N was not due to recurrent facilitation of the PBSt motoneurones

When the occurrence of recurrent effects on the Q Ia IPSPs in the Ib VSCT neurones was compared with the pattern of excitatory convergence onto the cells it was found that there was a clear specificity in the effects In all tested cells with the largest EPSPs from hip extensors or knee flexors the Ia IPSPs were susceptible to recurrent depression while no such effect was found in cells primarily excited from the knee ankle and toe extensors (Fig 5) All cells without recurrent depression of their Q Ia IPSPs received Ib EPSPs from the Q nerve although the EPSP in one cell was very small In the cells with affected Ia IPSPs on the other hand there were no Ib EPSPs from the Q nerve except a trace in two cells Interestingly the Q Ia IPSPs in the cells with convergence of Ia and Ib excitation from the hamstring nerves (*cf* section I) apparently were unaffected by the ventral root volleys

No other differences than in the pattern of Ib convergence were found between cells with and without recurrent effects on the Q Ia IPSPs The IPSPs had in both cases the same low threshold and similar central latencies and time courses In those few experiments in which the Q nerve was subdivided into the vastocrureus and rectus femoris branches cells were found with both types of IPSPs evoked from the vastocrureus branch The different effects are thus not to be related to the functional subdivision of the quadriceps muscle Further disynaptic Ia IPSPs from the Sart nerve were found in both groups of cells The effect of a ventral root volleys was however tested only with 2 such IPSPs in cells with affected Q Ia IPSPs and in both these cells the Sart Ia IPSPs were also depressed

In the Ib VSCT cells with affected Ia IPSPs the ... similar to

those found in Ia VSCT neurones (Gustafsson and Lindstrom 1973) or motoneurones (Hultborn *et al* 1971 a) The Q Ia IPSPs were depressed from the VRs L5 and L6 but not from the VRs L7—S1 and the depression had a latency and a time course similar to that in motoneurones In some cells disynaptic descending IPSPs and FRA IPSPs were also depressed following conditioning stimulation of the ventral roots and in the latter cases spatial facilitation between the Ia and the FRA volleys could be demonstrated All these observations indicate that the recurrently depressed Ia IPSPs in the Ib VSCT neurones were mediated by the same interneurones which relay Ia inhibition to motoneurones (*cf* Gustafsson and Lindstrom 1973)

## III *Disynaptic Ib IPSPs*

Eccles *et al* (1961) did not describe any disynaptic Ib IPSPs in the Ib VSCT neurones Such IPSPs have however been found in Ia VSCT cells (Lundberg and Weight 1971) and were therefore looked for very carefully in the present study In order to reveal even very small IPSPs the cells were usually strongly depolarized by current injection through the recording microelectrode and in addition small trains of maximal group I volleys were frequently used to obtain temporal facilitation in the Ib pathway Under these conditions disynaptic Ib IPSPs were found in the Ib VSCT cells although less frequently than Q Ia IPSPs They were usually also considerably smaller in amplitude than the Q Ia IPSPs even when a short train of nerve volleys was used

*Effects from nerves not giving Ib excitation to the cells* Disynaptic Ib IPSPs were evoked only in seven Ib VSCT neurones from nerves displaying separation of the incoming volley into Ia and Ib components Three of these cells were Sart Ib cells (without Ib EPSPs from the Q nerve *cf* Fig 2 D) which received disynaptic Ib IPSPs from the Q nerve The same cells also received disynaptic group I IPSPs from the Add nerve In another Sart Ib cell (with a Q Ib EPSP) a disynaptic Ib IPSP was evoked from the PBSt nerve Small Ib IPSPs from the PBSt nerve were also evoked in two cells with their largest Ib EPSPs from the Q nerve and one ABSm Ib IPSP was found in a G S cell These IPSPs had segmental latencies varying between 1 6 and 2 0 ms An example of a Q disynaptic Ib IPSP in a Sart Ib cell is shown in Fig 6 A—C There was no trace of an IPSP at a stimulus strength almost maximal for Ia fibres (A) but an IPSP appeared when the stimulation was increased to activate also some Ib fibres (B) and increased further when all group I fibres were stimulated (C)

Disynaptic group I IPSPs were more frequently evoked from one or more of the G S Pl FDL nerves (18 cells) Similar IPSPs are quite common in VSCT neurones lacking group I excitation (Lundberg and Weight 1971) and they occur also in Ia VSCT neurones (Lundberg and Weight 1971 Gustafsson and Lindstrom 1973) Since these nerves usually give rise to a single contoured group I nerve volley the Ia/Ib nature of these IPSPs could not be assessed for certain However the IPSP grew mainly in the higher group I range and were therefore presumably evoked by

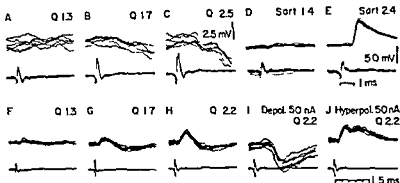



Fig 6 *IPSP evoked from Ib afferents in Ib VSCT neurones* A—E and F—J are from two different cells A—C disynaptic IPSPs evoked by Ib afferents in the Q nerve D—E monosynaptic EPSPs from Ib afferents in the Sart nerve F—H polysynaptic Ib IPSPs superimposed on the decay phase of monosynaptic EPSPs from the Q nerve The records in I and J show the effects of stimulation of the Q nerve with the same strength as in H but during injection of a strong depolarizing (I) or hyperpolarizing (J) current through the recording microelectrode Voltage calibration in C refers to intracellular responses in A—C and F—J voltage calibration in E refers to responses in D—E The time calibrations in E and J are for A—E and F—J respectively Further details as in Fig 1

Ib afferents (*cf* Lundberg and Weight 1971) They were found in all six groups of VSCT neurones shown in Fig 2 most frequently in the Sart (5/7) and Q (6/17) groups Disynaptic group I IPSPs were evoked also from the DP nerve in 7 G S and Q Ib VSCT cells all with relatively large Ib EPSPs from one or more of the G S Pl or FDL nerves These DP IPSPs were presumably evoked also from Ib afferent since they grew mainly in the higher group I range

*Convergence of monosynaptic excitation and disynaptic inhibition from Ib afferents in the same nerve* Some VSCT neurones receive convergence of monosynaptic excitation and disynaptic inhibition from the same group of Ia afferents (Lundberg and Weight 1971 Gustafsson and Lindstrom 1973) and this finding has formed the basis for the input-output comparator hypothesis for the VSCT (Lundberg and Weight 1970 Lundberg 1971) In view of this hypothesis it was of great interest to learn if the corresponding type of convergence occurs also in some Ib VSCT neurones

In the spinal preparation the transmission in the Ib pathways to motoneurones is usually rather weak (Eccles *et al* 1957 b Hongo *et al* 1969) and when present the Ib IPSPs are often quite small If the same interneurones which relay Ib inhibition to motoneurones terminate also on Ib VSCT cells as postulated in the comparator hypothesis one might expect the IPSPs to be similarly small in the VSCT neurones and to manifest themselves only as a deflection in the decay phase of the Ib EPSPs The detection of any such IPSPs in the VSCT cells was complicated by the fact that spikes frequently were generated already by EPSPs being a fraction of the maximal Ib EPSPs in the cells Hyperpolarizing current could not be used to stop this firing since any small Ib IPSPs would remain undetected when the membrane potential was moved towards the equilibrium potential for the IPSP To depolarize the cells

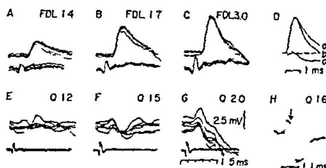



*Fig 7 Convergence of a monosynaptic EPSP and of a disynaptic IPSP from Ib afferents in the same nerve* A—D and E—H are from two different cells A—C monosynaptic EPSPs evoked by stimulation of the FDL nerve In B and C there are indications of an IPSP superimposed on the decay phase of the EPSP D superimposed tracings of the responses in C (trace b) and in A (trace a) the latter with the amplitude normalized to the same size as the EPSP in C The difference between the two curves is shown by the trace c The traces in D were obtained with a calculator having a digitizer input and an X—Y plotter output. E—H monosynaptic EPSPs and disynaptic IPSPs evoked by stimulation of the Q nerve in the Ib range The onset of the IPSP is seen in the average records (H) as a break in the falling phase of the EPSP (arrow) Voltage calibration in G refers to intracellular responses in A—G The time calibrations in D and G are for records in A—D and E—G respectively Further details as in Fig 1

drastically in order to block the spike generation mechanism was neither of any help since the interpretation of the findings was complicated by the frequent occurrence of local responses on top of the EPSPs

In spite of these difficulties indications of Ib IPSPs superimposed on the decay phase of the Ib EPSPs were found in some cells as exemplified in Fig 7 A—D A small EPSP with an almost exponential decay was evoked from the FDL nerve at a stimulus strength of 1 4 times threshold (A) When the stimulus strength was increased to 1 7 times threshold the first indication of a more rapid decay of the EPSP appeared (B) and this change became more apparent when stimulation was further increased to a strength maximal for group I fibres (C) The difference between the time course of the decay of the EPSP in A and C is most easily seen from the tracings in D where b corresponds to the EPSP in C and a to the EPSP in A The latter is plotted with the same time scale as in A but with the amplitude normalized to the size of the EPSP in C The difference between the two curves (c) would represent an IPSP superimposed on the decay of the EPSP in C Since the two curves a and b start to deviate about 0 7 ms after the beginning of the monosynaptic EPSP the first part of this IPSP should be evoked through a disynaptic pathway There is probably also a polysynaptic IPSP which might have been evoked from either Ib or group II afferents

The cell of Fig 7 received smaller Ib EPSPs from Pl G S and Q nerve and similar effects were evoked from the first two nerves while a disynaptic Ia IPSP from the Q nerve hindered the detection of any Ib IPSP from this nerve Results equivalent to the above were found in 7 other cells with the Ib effects from one or more of the Q PBSt ABSm G S Pl and FDL nerves Attempts were made to increase the amplitudes of the presumed Ib IPSPs by using a short train of group I volleys (3—5 stimuli with 3 ms intervals) Although the EPSPs evoked by the later

volleys in such a train invariably had a much more rapid decay than the first EPSPs the interpretation of the e results was complicated by the occurrence of small poly synaptic IPSPs in the cells (cf ection IV) When the cell of Fig 7 was hyper polarized by current injection through the recording electrode the time course of the decay of a maximal group I EPSP from the FDL nerve approached the time course of the decay of the 1 4 times threshold EPSP Similar results were found in the other cells but in no case it was possible to pass current enough to get a clear reversal of the response

A few cells received convergence of very small monosynaptic EPSPs and of di synaptic IPSPs which both grew within the Ib range An example is shown in Fig 7 E—H An almost maximal Ia volley evoked no effect in the cell (E) but a small monosynaptic EPSP and an IPSP appeared with the Ib volley (F—G) The segmen tal latency of the IPSP seemed to be about 0 8 ms longer than the latency of the EPSP as indicated by the break in the decay phase of the EPSP in the averaged records (H arrow) The IPSP should thus be disynaptic In view of the very small EPSP it is difficult to know if cells like this one can be ascribed any role as input output comparators Thus even if the pre ent results indicate that convergence of monosynaptic EPSPs and disynaptic IPSPs from Ib afferents in the same nerve does occur in VSCT neurones as postulated in the comparator hypothesis (Lundberg 1971) final proof of these connextions probably has to await experiments in which the tran mission in the Ib pathways can be facilitated from some descending tracts

IV *Polysynaptic Ib IPSPs*

IPSPs which grew within the Ib range and had segmental latencies indicating a tri or polysynaptic linkage were ob erved in some Ib VSCT neurones by Eccles *et al* (1961) and such IPSPs are common also in other VSCT neurones (Lundberg and Weight 1971 Gustafs on and Lindstrom 1973) The afferent origin of these IPSPs is not certain although it has generally been assumed to be Ib afferents (Eccles *et al* 1961 Lundberg and Weight 1971) Similar IPSPs have been observed in 29 cells in the present study This is probably an underestimated figure since these IPSPs are often difficult to differentiate from polysynaptic group II IPSPs which are evoked in practically all VSCT neurones Only ca es in which the IPSPs appeared near threshold for the Ib volley or at a very low group I strength have been included The differentiation between these IPSPs and polysynaptic group II IPSPs was, however not entirely based on threshold differences for electrical stimulation It was not unusual to find that the polysynaptic IPSPs increased in amplitude to a plateau at a stimulu strength just maximal for group I fibres and further increase was not obtained until the stimulus strength was enhanced to some 1 5—2 times this value This may indicate that the polysynaptic group II IPSPs were evoked through a eparate group of interneurones requiring a certain amount of spatial facilitation In these cases the group II IPSPs al o eemed to have slightly longer latencies than the IPSPs evoked within the group I range Polysynaptic group Ib IPSPs were most frequently evoked from the Q nerve but similar effects were found also from the

Sart PBSt ABSm G S Pl and FDL nerves They were found in all groups of Ib VSCT neurones and occurred without other group I effects from the same nerve or in combination with disynaptic Ia or Ib IPSPs (*cf* prolongation of IPSPs in Fig 4 G—H and Fig 6 B—C) or monosynaptic Ib EPSPs An example of the latter type of convergence is shown in Fig 6 F—J There was a certain overlap between the Ia and Ib volleys from the Q nerve in this cat The small EPSP in F therefore presumably was evoked from Ib afferents, which contaminated the Ia volley An IPSP appeared at a stimulus strength of about 1 4 times threshold and it had an appreciable amplitude at 1 7 times threshold (G) and was slightly further increased at maximal group I strength (H) The large increase in amplitude of this IPSP when the cell was strongly depolarized by current injection through the recording electrode is shown in I and the reversal of the IPSP by a strong hyperpolarizing current in J It was often very difficult to measure accurately the central latencies of these IPSPs when they were superimposed on other EPSPs or IPSPs However in several cells as the one in Fig 6 the latencies were between 2 4—3 0 ms indicating a trisynaptic linkage for the effect

## Discussion

The properties of VSCT neurones monosynaptically excited from Ib afferents have been extensively studied by Oscarsson and colleagues (Oscarsson 1956 1957 1960 Eccles *et al* 1961 Magni and Oscarsson 1961 Lundberg and Oscarsson 1962) In overlapping parts the present results are in good agreement with the earlier findings Thus most individual cells received Ib excitation from many nerves and the pattern of convergence was similar to that found earlier When analyzing these convergent effects quantitatively it was, however apparent that most cells received their dominating excitation from only one or a few muscles acting as synergists at the same joint Groups of cells mainly connected with either flexor or extensor muscles acting at the hip and knee joints or with extensor muscles of the ankle joint were found Since Eccles *et al* (1961) found a few cells exclusively excited from the nerve to the pretibial flexor muscles and one cell mainly excited from the nerve to the toe extensor muscles was found in the present experiments flexor and extensor muscles of all joints in the hindlimb seem to be represented With the exception of the close parallelism in effects from Ib afferents of the hip extensor ABSm and knee flexor PBSt muscles the additional excitation of the Ib VSCT neurones seemed mainly to originate from other extensor muscles in cell with their largest EPSPs from extensor muscles and from flexor muscles in flexor cells The convergence of excitation from the ABSm and PBSt nerves may be related to the fact that the AB and PB nerve branches supply two functionally different parts of the same biceps muscle (*cf* Results section I) From a quantitative point of view the pattern of excitation of the Ib VSCT neurones thus seems to be functionally more specific than could be infered from earlier studies (Oscarsson 1957 Eccles *et al* 1961) or from observations of individual cells Interestingly there seems also to be a special

group of VSCT neurones which receives convergence of monosynaptic excitation from Ia and Ib afferents (*cf* also Eccles *et al* 1961 Lundberg and Weight 1971)

Confirming the results of Eccles *et al* (1961) disynaptic Ia IPSPs from the Q nerve (to a lesser extent also from the Sart nerve) were found to be common in all groups of Ib VSCT neurones except cells excited mainly from the Sart nerve Some of the latter cells instead received disynaptic Ib IPSPs from the Q nerve Surprisingly there was no evidence of Ia IPSPs from any other dissected nerves Undubious disynaptic Ib IPSPs from thigh nerves were rare while disynaptic group I IPSPs presumably evoked from Ib afferents were relatively common from nerves to the ankle and toe muscles G S Pl FDL and DP IPSPs growing within the Ib range and having central latencies indicating a tri or polysynaptic linkage were also common especially from the Q nerve

Considering the input-output comparator hypothesis for the VSCT (Lundberg and Weight 1970 Lundberg 1971) it was of great interest that indications of convergence of monosynaptic excitation and disynaptic inhibition from Ib afferents in the same muscle nerve were found in some VSCT neurones as has earlier been found with respect to effects from Ia afferents (Lundberg and Weight 1971 Gustafsson and Lindström 1973) The fact that the presumed IPSPs were small is not in contradiction to the hypothesis since Ib IPSPs in motoneurones often are quite small (Eccles *et al* 1957 b Hongo *et al* 1969) Final proof of the indicated convergence however has to await future experiments in which it would be possible to facilitate the transmission in the Ib pathways for instance from the rubrospinal tract (Hongo *et al* 1969) Since many Ib IPSPs in motoneurones are evoked through a trisynaptic pathway (Eccles *et al* 1957 b) especially from the Q nerve (Lundberg personal communications) it is possible that VSCT neurones with convergence of monosynaptic excitation and trisynaptic inhibition from Ib afferents in the same nerve (*cf* Fig 6) represent a special type of input-output comparators They may receive collateral connextions from the excitatory Ib fibres impinging on the first order interneurones in the Ib pathway and from the inhibitory last order interneurones terminating on motoneurones (*cf* also Lundberg 1971)

One of the most interesting results of the present study is the finding that the Ia IPSPs in some Ib VSCT neurones were recurrently depressed by impulses in motor axon collaterals just as in Ia excited VSCT neurones (Gustafsson and Lindström 1973) and motoneurones (Hultborn *et al* 1971 a) while similar Ia IPSPs in other Ib VSCT neurones were unaffected by the ventral root volleys This finding is even more striking when considering that both types of Ia IPSPs were evoked from afferents in the same nerve (Q) and did not seem to differ in any other respect The result can hardly be due to experimental failures Cells without effect were found in several preparations and were intermixed with cells in which the Ia IPSPs were depressed It was also carefully controlled by doing many conditioning testing runs with test Ia IPSPs of different amplitudes that small recurrent effects were not overlooked Further it was regularly checked on monosynaptic test reflexes that the used ventral root volleys were effective in evoking recurrent inhibition in Q Ia inhib t

interneurones terminating on PBSt motoneurones The two types of Ia IPSPs were found in a strictly organized pattern in relation to the Ib excitation of the VSCT cells The Ia IPSPs in cells with the largest Ib EPSP from either the PBSt Gnac or ABSm nerves were all recurrently depressed while no such effect was found on any Ia IPSP in VSCT neurones with the largest Ib EPSP from other nerves It seems necessary to accept that the Ia IPSPs in these two groups of Ib VSCT neurones were mediated through two different types of Ia inhibitory interneurones one with recurrent inhibition from motor axon collaterals the other without (*cf* Fig 1 Gustafsson and Lindstrom 1973) This is a new example of the highly differentiated convergence onto VSCT neurones (*cf* Lundberg and Weight 1971 Lindstrom and Schomburg 1973) and demonstrates that similar postsynaptic potentials from the same group of afferents in VSCT neurones cannot a priori be assumed to have the same functional significance

This finding is also the first demonstration of disynaptic Ia IPSPs unaffected by impulses in recurrent motor axon collaterals and thus of the existence of Ia inhibitory interneurones lacking recurrent inhibition Similar results have now been obtained with respect to disynaptic Ia IPSPs in dorsal spinocerebellar tract neurones (Lindstrom and Takata to be published) and in some spinal interneurones (Lindstrom and Schomburg unpublished) The effects in the Ib VSCT cells with recurrently depressed Ia IPSPs were similar to those obtained in Ia VSCT neurones (Gustafsson and Lindstrom 1973) and motoneurones (Hultborn *et al* 1971 a Hultborn 1972 a) regarding the time course and effective ventral root for the recurrent depression and the interaction with the FRA It is thus likely that these IPSP were mediated through collaterals of the interneurones which mediate Ia inhibition to motoneurones (*cf* Gustafsson and Lindstrom 1973) Since all evidence indicates that motoneurones are inhibited by a homogeneous population of Ia inhibitory interneurones all receiving recurrent inhibition (Hultborn *et al* 1971 a b Hultborn 1972 a) the unaffected Ia IPSP in the Ib VSCT cells should be evoked from interneurones not having direct access to motoneurones Possible candidates are the interneurones in the intermediate region of the spinal cord which receive monosynaptic excitation from Ia afferents but no recurrent inhibition (Hultborn *et al* 1971 b) and which originally were assumed to relay Ia inhibition to motoneurones (Eccles *et al* 1956 *cf* Hultborn *et al* 1971 b) The present finding that susceptibility to recurrent inhibition from motor axon collaterals is not a general property of Ia inhibitory interneurones emphasizes the selectivity in the recurrent control of the interneurones which relay reciprocal Ia inhibition to motoneurones (Hultborn *et al* 1971 a Hultborn 1972 a and is also of importance for the evaluation of the functional significance of the Ia IPSP in VSCT neurones (*cf* Gustafsson and Lindstrom 1973 Lindström 1973)

Several observations indicate that the VSCT rather than being an afferent relay pathway signals information about central events at the segmental level in the spinal cord For example many VSCT neurones lack monosynaptic excitation from primary afferents (Lundberg and Weight 1971 *cf* also Eccles *et al* 1961 Lundberg

and Oscarsson 1962) Instead these cells frequently receive monosynaptic excitation from descending tracts which are known to have monosynaptic connexions with segmental interneurones (Lundberg and Weight 1971 Baldissera and Bruggencate 1969 Baldissera and Weight 1969) VSCT cells excited from primary afferents differ from other ascendin tract neurones e g dorsal spinocerebellar tract cells in being very weakly driven by adequate activation of appropriate receptors (Oscarsson 1960) Further Arshavsky *et al* (1972) found that the rhythmic modulation of the impulse discharges in VSCT neurones in walking cats remained after deafferentation The recurrent inhibition from motor axon collaterals of some VSCT neurones (Lindstrom and Schomburg 1973) and the recurrent control of the Ia inhibitory pathway to VSCT neurones (Gustafsson and Lindstrom 1973) are also difficult to interpret in terms of an afferent relay pathway All the e results are however in accordance with the hypothesis that the VSCT relays information about the transmission in interneuronal reflex pathways to motoneurones (Lundberg 1971) It seems therefore reasonable to interpret the present findings regarding the Ib VSCT neurones in the same terms

According to the above hypothesis the VSCT neurones receive information about he activity in specific reflex pathways to motoneurones through collateral connexions from neurones which terminate on the interneurones in the reflex pathway of interest (Lundbrg 1971) If this hypothesis is generally valid then the found convergence onto the Ib VSCT neurones would have its counterpart in the organization of the Ib pathways in the spinal cord and may thus give insight into the organization of these pathways Although Lundberg (1971) discussed the VSCT function mainly in relation to inhibitory pathways to motoneurones also excitatory Ib pathways may be concerned (*cf* Lindstrom 1973) Unfortunately the organization of the Ib pathways to motoneurones is not known enough to allow a detailed comparison with the pre ent findings from Ib VSCT neurones (*cf* Laporte and Lloyd 1952 Eccles *et al* 1957 b Hongo *et al* 1969) On a superficial level the wide convergence of Ib effects from different extensor nerves in motoneurones (Eccles *et al* 1957 b) is matched by a similar wide convergence of Ib excitation from extensors in the VSCT neurones Whatever the reason for the frequent convergence of excitation from the PBSt nerve in exten or Ib VSCT neurones (*cf* Results) this convergence has also its counterpart in the Ib effects on motoneurones since the PBSt nerve together with extensor nerves supply Ib inhibition to G S motoneurones and Ib excitation to DP motoneurones (Eccles *et al* 1957 b Table 1) It might be postulated that the convergence of disynaptic inhibition in the Ib VSCT neurones from Ia afferents and from Ib afferents in nerves not supplying Ib excitation to the cells reflects an inhibitory control of the interneurones in Ib pathways to motoneurones from other Ia and Ib reflex pathways Interactive connexions between different segmental reflex pathways are presumably common (Lundberg 1966 1969 Hultborn 1972 b) although o far nothing is known about the Ib pathways in this respect The possibility of an interactive control of the Ib pathways from other Ib and Ia pathways has however been discu sed in relation to observations from Ib interneurones in the intermediate region

(Hongo *et al* 1972) Although the terminations of these cells are unknown there is reason to believe that at least some of them mediate Ib effects to motoneurones (Hongo *et al* 1972) Disynaptic group I IPSPs are common in these cells (Hongo *et al* 1966 1972) and some of them seem to be evoked from Ia afferents in the Q nerve (Jankowska and Lundberg personal communications) Intermixed with the Ib interneurons there are other interneurones monosynaptically excited from Ia afferents (Eccles *et al* 1956 1960 Hongo *et al* 1966 1972) but lacking recurrent inhibition from motor axon collaterals (Hultborn *et al* 1971 b) In other interneurones in the intermediate region there seems to be a convergence of monosynaptic excitation from Ia and Ib afferents in the same nerve (Hongo *et al* 1966 *cf* also Fig 32 in Eccles 1964) just as in some of the VSCT neurones found in the present study The latter two types of interneurones have been suggested to have an inhibitory role in the interactive control of the Ib pathways (Hongo *et al* 1972) Some of the VSCT neurones may thus convey information about interneurones having an interactive role in the spinal cord In view of the speculative character of these considerations it is not meaningful to discuss presently why some of the Ib pathways to motoneurones should receive inhibitory control from the interneurones which mediate Ia inhibition to motoneurones while other Ib pathways should receive Ia inhibition not influenced from motor axon collaterals and Renshaw cells as suggested by the present findings in the Ib VSCT neurones However since the Ia inhibition of the Ib VSCT neurones seems to occur in a consistent pattern the suggestion that this inhibition reflects an inhibitory control of the Ib pathways to motoneurones should be possible to test experimentally

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(Hongo *et al* 1972) Although the terminations of these cells are unknown there is reason to believe that at least some of them mediate Ib effects to motoneurones (Hongo *et al* 1972) Disynaptic group I IPSPs are common in these cells (Hongo *et al* 1966 1972) and some of them seem to be evoked from Ia afferents in the Q nerve (Jankowska and Lundberg personal communications) Intermixed with the Ib interneurons there are other interneurones monosynaptically excited from Ia afferents (Eccles *et al* 1956 1960 Hongo *et al* 1966 1972) but lacking recurrent inhibition from motor axon collaterals (Hultborn *et al* 1971 b) In other interneurones in the intermediate region there seems to be a convergence of monosynaptic excitation from Ia and Ib afferents in the same nerve (Hongo *et al* 1966 *cf* also Fig 32 in Eccles 1964) just as in some of the VSCT neurones found in the present study The latter two types of interneurones have been suggested to have an inhibitory role in the interactive control of the Ib pathways (Hongo *et al* 1972) Some of the VSCT neurones may thus convey information about interneurones having an interactive role in the spinal cord In view of the speculative character of these considerations it is not meaningful to discuss presently why some of the Ib pathways to motoneurones should receive inhibitory control from the interneurones which mediate Ia inhibition to motoneurones while other Ib pathways should receive Ia inhibition not influenced from motor axon collaterals and Renshaw cells as suggested by the present findings in the Ib VSCT neurones However since the Ia inhibition of the Ib VSCT neurones seems to occur in a consistent pattern the suggestion that this inhibition reflects an inhibitory control of the Ib pathways to motoneurones should be possible to test experimentally

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pigs were anesthetized with chloralose 1 % and urethane 3.5 % 3 ml/kg Twenty subsequent guinea pigs and all ten squirrels were given dial 20 mg/kg Ether was given by inhalation as necessary during the operation

The animals were intubated and the left visual cortex was exposed The dura was left intact The animals were fixed in a small animal head holder and the skin around the craniotomy was sewed to a ring to make a pouch that was filled with mineral oil. The temperature measured with a thermistor close to the brain was kept at 35—37 °C by an infra red lamp above the head The rectal temperature was kept at 37° by a heating pad

The light source was a gas flash vacuum tube (VTE or G 419 Erlangen) discharged from a charged condenser (20 µF) across an inductance (1 mH) and a rectifier The condenser was recharged over an inductance (100 mH) from a 315 V supply The flashes lasted less than 1 ms and were delivered once in 10 s Paired flashes were obtained by triggering the generator from a stimulator (Disa Multistim) The shortest interval between paired flashes was 15 ms and the intensity of the conditioning and test flashes was the same

The light was collimated passed through an aperture to give white light or through Balzer interference filters with peak transmission at 450 505 550 and over 605 nm The energy of the flashes was measured with a vacuum photomultiplier tube (Buchmann Olsen and Rosenfalck 1957) by comparison with a calibrated standard bulb The intensity of the light was varied with grey filters each of which transmitted 10 % of the light

The light was focused onto the right cornea to illuminate the eye in Maxwellian view The pupil was dilated with 1 % homatropine A shield protected the eye from light other than from the flash the left eye was covered and the room was illuminated only by the infrared lamp above the head At least 1 h of dark adaptation preceded each series of flashes

*Recording* The electroretinogram was recorded with a silver wire that encircled the eye at the corneal junction the remote electrode was a steel cannula thrust into the skin over the nose The cortical potential was recorded with a silver ball suspended on a light spring and placed on that point on visual cortex where the response was largest The indifferent electrode was a silver ball on the bone at the anterior edge of the craniotomy The animal was grounded by a steel needle inserted through the skin over the back

The two difference amplifiers had an input impedance of 200 MΩ shunted with 30 pF (balanced) a lower limiting frequency of 3 Hz (3 dB down) and an upper limiting frequency of 10 000 Hz (3 dB down) both with a slope of 6 dB/octave The noise level of the amplier with short-circuited input was 3 µV r.m.s The responses were displayed on a double beam oscilloscope (Disa) and the sweeps were photographed on 35-mm film with a Dumont camera The stimuli were signalled with a marking pulse from the generator and a photocell displayed the flash

*Procedure to determine the recovery time* In the case of squirrels double flashes were delivered in 10 ms steps from 15—20 to 80 ms between flashes in 20 ms steps from 80 to 120 ms between flashes and then in 50 ms steps to 300 ms between flashes In the case of guinea pigs the intervals between flashes were 60 to 3000 ms and the steps 20 to 250 ms

Each flash pair was repeated 5 times at a rate of 1 per 10 s and was recorded either superimposed on the same sweep or on separate sweeps A full series took about 3 h

*Measurement* The responses were measured from film the pictures enlarged 6 times

## Results

*A Guinea pigs*

1) *Retinal responses* were small and variable and no reliable results were obtained

2) *Cortical responses to the first of paired flashes* The intensity of the light was so high that cortical responses to blue and red flashes were obtained over 3—4 decades The summed cortical response to the first of paired flashes was diphasic with an initial positive phase After flashes of maximum intensity the response appeared after a latency of 30 ms and had an amplitude of up to 2000 µV Some 120 to 160 ms after each cortical response there was a second response much more variable in size and latency than the first and not always present It could conceal or distort a response to the second flash when the two responses coincided

*Changes in amplitude with intensity* The amplitude increased nearly linearly with the intensity of the flashes averaging 300 µV for each ten fold increase in

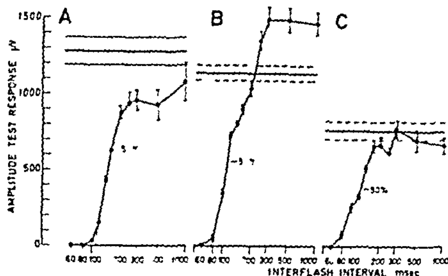



Fig. 1. Amplitude/time relations of cortical responses to < 1 ms red flashes in guinea pigs under chloralose urethane anesthesia. Amplitude of test responses (ordinate) as a function of interflash interval (abscissa). The bars show the mean error and the hatched areas show the amplitude and the mean error of the response to the conditioning flash. The interval time when the test response is 50 % of full amplitude is drawn on the curves. A. Flashes of maximum intensity. B. Flashes 10 and 1 of maximum intensity (no difference between them). C. Flashes 0.1 of maximum intensity (near threshold).

in animals anesthetized with chloralose urethane 150 µV per ten fold increase in intensity of the light when dial was the anesthetic.

In both groups of animals there was no further increase in amplitude when the light was increased from 10 % of maximum to maximum, indicating that the highest intensity was supramaximal.

*Change in amplitude with color.* At each intensity step except one the responses to red and to blue flashes had the same amplitude. At the only intensity step where there was a difference responses were larger to blue flashes in dial anesthesia and to red flashes in chloralose urethane anesthesia, suggesting that the difference was fortuitous.

3) *Cortical responses to the second of paired flashes.* a) *Chloralose urethane anesthesia* (Fig. 1). When the anesthesia was chloralose urethane the response to the test flash began to appear when the second flash followed the first by 80 to 120 ms; the amplitude of the test responses increased nearly linearly with time up to 120 to 160 ms between flashes. The absolutely irresponsive time was found by extrapolating the linear increase in amplitude back to the baseline. It was about 110 ms after the most intense flashes.

Since the intensity of the conditioning decreased as did the intensity of the testing flash the true refractory time was not measured. As the flashes decreased in strength responses to the second flash appeared earlier and increased in size faster (Fig. 1). The resultant decrease in recovery time to 70—80 ms indicated that elements were

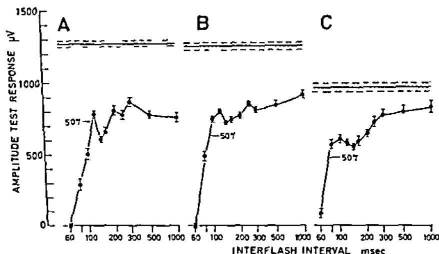



Fig 2 Amplitude/time relations of cortical responses in guinea pigs under dial anesthesia The flashes and symbols are the same as in Fig 1

available to respond to the second flash that had not been activated by the first sub maximal one

With the weakest blue flashes the recovery time rose again to close to 90 ms probably reflecting a real increase in recovery time with decreased intensity of the stimulus There was no difference of responses to blue and to red flashes when the responses had the same amplitude

The time to full recovery was prolonged after the most intense flashes in that the response to the second flash was still not as large as to the first at the end of a second With diminishing intensity the second response became as large as the first at progressively shorter interval With flashes of submaximal intensity the response to the second flash exceeded the first in size when the flashes were separated by 200—300 ms or more

II) *Dial anesthesia* (Fig 2) In animals anesthetized with dial, there was usually no response to the second flash at interflash intervals of 60 ms whereas with 80 ms between flashes the mean response to the second flash was one third to one half the size of the first Thus the recovery time lay somewhere between 60 and 80 ms intervening intervals were not tested Just as the absolutely irresponsive time was shorter in dial than in chloralose urethane anesthesia so was the time when the test response reached 50 % of the amplitude of the conditioning response shorter in dial anesthesia by as much as 40—50 ms (Fig 1 and 2 A and C) With diminishing intensity of the flashes the time to 50 % amplitude tended to diminish with both forms of anesthesia

*Time to full recovery* on the other hand was much longer in dial anesthesia After the initial abrupt increase in amplitude of the test response with increasing interflash intervals the amplitude of the test response tended to fall or flatten out and then increased so slowly that it had not approached the range of amplitude of

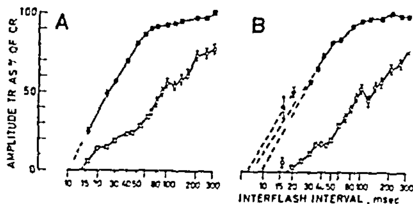



Fig. 3. Amplitude/time relations of retinal (●—●) and of cortical (○—○) responses in squirrel under dial anesthesia. Ordinate: amplitude of the test response as % of the conditioning response. Abscissa: interflash intervals in milliseconds. The bars give the mean error. A. The flashes gave retinal and cortical responses of the same high mean amplitude (66.5 and 90.3 µV) (white 10% of maximum, green (505 and 550 nm) at maximum intensity). B. As in A, but the mean amplitudes were smaller 43.9 and 61.4 µV (green 10% of maximum (505 and 550 nm) and red and blue of maximum intensity).

the conditioning response when the interflash intervals were 1000 ms except when the flashes were weak (Fig. 2C).

B. *Ground squirrels* (all under dial anesthesia).

1) *Retinal responses* (Fig. 3). The threshold was high, and only white and green flashes could be used at maximum and at 10% of maximum intensity. At 1% of maximum intensity the responses were not reproducible, and at maximum intensity the white flashes were supramaximal, since the amplitude was lower and the recovery time longer than at 10% maximum intensity.

Since the effect of changing the intensity of the flashes could not be determined and since there was no consistent difference with color, the curves in Fig. 3A are the mean of those retinal and cortical responses of about the same high amplitude and those in Fig. 3B are the mean of the reproducible responses of somewhat lower amplitude.

There was always a response to the testing flash with the shortest interflash intervals available, 15 ms, and the test response had reached 50% of the amplitude of the conditioning response when the interval between flashes was 25 ms.

The amplitude of the response to the test flash increased linearly with increasing interflash intervals to 50 ms and then increased more slowly. Extrapolation of the linear portion of the curve back to the baseline gave an absolutely irresponsive time on the order of 10 ms, possibly less for the smaller responses (Fig. 3). The time to full recovery was longer for the larger responses: the test response reached full amplitude when the interflash interval was 300 ms (Fig. 3A), whereas the test response was as large as the smaller conditioning response when the interval was 100 ms (Fig. 3B). After white flashes of maximum intensity the time to 50% amplitude was somewhat longer, 37 ms.

2) *Cortical responses* The abrupt positive response began some 10 ms after the flash and had a duration of about 40 ms It was followed by a variable positive response starting about 100 ms after the flash and ending at about 160 ms The response to the test flash could be difficult or impossible to find as it coincided with the second conditioning response but it could usually still be identified when the flashes were separated by 15—20 ms even though it then fell on the descending limb of the first response Thus the irresponsive time of the cortical response was often not identified with the shortest interflash interval used

The *initial increase in* amplitude of the response to the test flash with increasing interflash interval was linear extrapolation back to the baseline gave absolute irresponsive times on the order of 15 ms *i e* at least 5 ms longer than the irresponsive time of the retinal response The time to 50 % amplitude was on the order of 100 ms or 4 times that of the retinal response

When the interflash interval was 300 ms the amplitude of the cortical response to the test flash was 70—80 % of that to the first flash suggesting that also the time to full recovery was shorter in the cortex of squirrel than of guinea pig under the same anesthesia

## Discussion

The cortical recovery time was much shorter in an animal with a cone eye the ground squirrel *Citellus citellus* than in an animal wih a rod eye albino guinea pig under the same conditions In the squirrel there was a response to the test flash when the interflash interval was 15 ms in the guinea pig the response to the test flash was rarely present when the interflash interval was 60 ms anesthesia in both animals being dial

In the guinea pig the retinal responses were small and variable and the cortical recovery time could be compared with the retinal only indirectly by comparing the cortical refractory time after bright flashes and after shocks to the optic nerve The conclusion was that the refractory times of the main cortical and of the retinal response were equal (Simon en 1969)

In the squirrel on the other hand the retinal responses were large and re producible and the retinal recovery time of 10 ms was clearly shorter than the corti cal 15 ms The amplitude of the respon e to the test flash was 50 % of that to the *conditioning flash at an interval of 25 ms for the retinal and of 100 ms for the corti* cal response Thus in a cone animal it is the cortex that limits the temporal resolving power of the visual system The same finding has been reported in animals with rods and cones *i e* monkey (Walker *et al* 1943) and cat (Lindsley 1953 Grusser *et al* 1962 Brazier 1963)

Crescitelli and Gardner (1961) al o found a longer recovery time of cortical than of retinal evoked potentials in squirrel but the cortical recovery times were as long as or longer than the cortical recovery time in guinea pig in my study The experi mental conditions were different Anesthesia was with nembutal and the flashes were longer at least 20 ms The rate of repetition was not given Presumably the longer

duration of the flashes accounts largely for the much longer recovery time than I found.

Certainly the type and amount of anesthesia are also important factors. The period of elevated amplitude of the response to the test flash at certain interflash intervals that I found with chloralose urethane anesthesia (Fig. 1C) was described for chloralose anesthesia by Castaut *et al.* (1951). In my study dial though it lowered the irresponsive time compared with chloralose urethane depressed the amplitude of the test response for at least a second. This depression was not prominent in the study of Gastaut *et al.* (1951) although they gave to cats twice the dose per kg that I used for guinea pigs. Possibly there is a species difference. Without anesthesia the irresponsive time in cats was of the order of 20 ms by contrast with 80 ms with dial and 100 ms with chloralose in the same range I found with the same anesthetic agents in guinea pigs.

In cats with their mixed rod and cone retina central conduction time was faster when the flashes were red than when they were blue or green (Lennox 1956). Similarly single fibres in the optic tract with slow conduction velocity responded better to blue than to red flashes whereas fibres with fast conduction velocity responded as well to red as to blue (Lennox 1958). It seems possible that the large *difference in temporal resolving power of rod and of cone connections in the central* nervous system may explain that finding.

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# The Effect of Water Diuresis and Antidiuretic Hormone on the Regional Renal Red Cell Flow

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Abstract

PERSSON A E G J SCHNERMANN H R ULFENDAHL M WOLGAST and P WUNDERLICH *The effect of water diuresis and antidiuretic hormone on the regional renal red cell flow* Acta physiol scand 1974 *90* 193—201

The possible role of the renal medullary hemodynamics in the formation of hypertonic and hypotonic urine was examined in 9 mongrel dogs using an indicator-dilution technique with $^{32}P$ labelled red cells internal detection with small needle-shaped semiconductor detectors The red cell flow was estimated from
a) the mean transit time calculated by dividing the area of the indicator-dilution curve by the peak activity and
b) the regional red cell volume determined as the ratio of intrarenal equilibrium activity to red cell equilibrium activity in whole blood
In water diuresis as induced by 700—400 ml of a hypotonic saline glucose solution the red cell flow showed a small and barely significant increase estimated at 19 % 18 % and 10 % for the cortical outer medullary and inner medullary circulations respectively After the administration of antidiuretic hormone at a rate of 1 5 mU/min and kg the cortical and outer medullary red cell flows (but not the inner medullary) were significantly reduced towards the control values It is concluded that the conditions of water diuresis and antidiuresis as due to the action of ADH are not accompanied by marked changes in the renal medullary blood flow

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*The effect of water diuresis and antidiuretic hormone on the regional renal red cell flow*

It is generally accepted that increased plasma levels of antidiuretic hormone elicit functional changes resulting in the production of a more concentrated urine In 1967 Berliner and Bennet summarized 3 possible actions of this hormone as

1 increased water permeability in the distal nephron
2 increased sodium reabsorption from the loop of Henle and
3 reduced medullary blood flow

In the present investigation the last possibility has been examined by trying to

determine whether water diuresis and the injection of arginine vasopressin are accompanied by changes in the outer and inner medullary red cell flows. An indicator dilution technique has been used with injection of P labelled red cells into the renal artery and internal detection of the radioactivity by small needle shaped semiconductor detectors (Wolgast 1968). Previous studies made in this field have given controversial results. Thus Thurau, Deetjen and Kramer (1960) using a photoelectric technique found that the inner medullary blood flow was markedly increased during water diuresis and could be restored to control (antidiuretic) values by the injection of antidiuretic hormone (ADH). These results were confirmed by Fourman and Kennedy (1966) who used the degree of staining of a fluorescent dye in the vasa recta wall as an index of medullary blood flow. In contrast to the findings Aukland (1966) on measuring the wash out of inert diffusible gas was unable to find any important effect of vasopressin. It has been claimed (Thurau and Levin 1971) that an unchanged wash out rate of inert gas might be the result of an actually reduced flow in the vasa recta and an increased flow in the loop of Henle, the latter due to an increased juxtamedullary GFR as found by Horster *et al.* 1968.

## Material and Methods

Experiments have been carried out on 9 seemingly healthy mongrel dogs weighing between 13 and 4 kg. Anesthesia was induced by Pentothal sodium® Abbott 10 mg/kg and followed by chloralose 0.8 in saline or water in an initial dose of 60 mg/kg or less and additional small maintenance doses in the order of 0 mg/kg and hour. All dogs were tracheotomized to ensure free airways and breathed spontaneously. Polyethylene catheters were introduced into the cubital veins for infusions and into the femoral artery via a small branch for blood pressure recording and blood sampling. Via a flank incision and retroperitoneal dissection the ureter and the spermatic or ovarian vein were exposed and cannulated with silicon catheters; the spermatic catheter was then advanced about 1 cm into the renal vein. The renal artery was cannulated with a mandrin supplied nylon catheter (O.D. 0.5 mm) via a lumbar artery located opposite to the origin of the renal artery. No free dissection of the renal hilus region was carried out in order to keep the nervous supply as unaffected as possible.

The blood pressure was measured with a Statham strain gauge transducer and recorded on a photokymograph (Ultralette mod. ABEM, Sweden). The hematocrit was determined by centrifugation at 10 000 × g in a microcapillary centrifuge. The urine flow was measured with a simple drop counter or for more precise measurements with measuring cylinders, and its osmolality was determined by a freezing point depression method (Osmometer mod. LS Advanced Instr., Newton Highland 61, Ma., USA). The glomerular filtration rate (GFR) was determined as the clearance of creatinine and the total renal blood flow by the PAH extraction method.

The regional red cell flow was determined by the indicator dilution technique previously described (Wolgast 1968) with P labelled red cells as the red cell indicator and detection by means of small beta sensitive semiconductor detectors inserted into the parenchyma. For labelling ml of pure red cells were mixed with a small amount (~0.3 ml) of a citrate phosphate buffer solution (Mollison 1958) to which about 1 mCi of P phosphate had been added. The solution was incubated at 33 C for 1 h after which some 75 of the P had been incorporated into the red cells. The labelled cells were then washed three times with saline and suspended in their own plasma.

In the present series two 1.5 mm thick semiconductor detectors were used—one with three sensitive elements and the other with one element. As a rule one of the sensitive elements was placed in the cortical parenchyma, one in the outer medullary and two in the inner medullary parenchyma. The analyzing equipment consists of a 3 channel amplification system (AB Atomenergi, Sweden), the output signal being fed to the photokymograph. The time constants were set at 0.3, 1.0 and 3.3 s for the cortical, outer medullary and inner medullary recordings, respectively.

The regional red cell flow was calculated from a) the mean transit time as determined by dividing the area of the curve by the peak activity (see Wolgast 1968) and b) from the regional red cell volume as determined by the ratio of intrarenal equilibrium activity to the equilibrium activity of red cells in a blood sample withdrawn on the same occasion Changes in the regional red cell flow were thus calculated from the change in the mean transit time and change in the red cell volume

*Experimental procedure*

3 conditions were examined ri the control antidiuretic condition the water diuretic condition and the antidiuretic state obtained after infusion of ADH In all these experimental periods the regional red cell flow was investigated in duplicate or triplicate by the injection of 0 5 ml or less of the labelled red cells as a slug injection into the renal artery and recording of the indicator dilution curves for at least 3 min The regional red cell volume was usually determined in duplicate about 5 min after an injection of labelled red cells The GFR total renal blood flow urine flow and urine osmolality were estimated from two urine collection periods of 10—15 min

After the control period in estigation water diuresis was induced by a single injection of 100 ml of a solution containing 0 25 % NaCl and 4 % glucose followed by a continuous injection at a rate of about 2 ml/min In most cases steady water diuresis was obtained after 1.5 h and the total amount of fluid injected then amounted to 200—400 ml To check the effect of anesthesia on the possibility of inducing water diuresis one dog was anesthetized with Nembutal in which case 1500 ml had to be infused in order to obtain a hypotonic urine

After a new set of blood flow determinations arginine asopressin as infused at a rate of 1 5 mU/min and kg except for 1 expt (No 7) where 3 mU/min and kg was infused

## Results

In Fig 1 two sets of indicator dilution curves (exp No 1) as obtained during water diuresis (left figure) and after the infusion of ADH at a rate of 1 5 mU/min and kg are presented The short time lapse of the cortical recordings contrast here with the prolonged curves obtained in the medullary parenchyma The initial notch seen in the outer medullary recording is due to the passage of indicator through arcuate arteries included in the periphery of the monitored volume The urine flow fell from 2 ml/min to about 1 ml/min when the latter curves were obtained The osmolality increased at the same time from 180 to 520 mOsm/kg It is clearly seen that the transit time in the cortical (III) and the outer medullary recording (I) did not change essentially In the inner medulla (II) the transit time was increased from 15 4 to 19 4 s this will mean a 20 % decrease in the reciprocal of the mean transit time The corresponding values for the cortex and outer medulla were 7 % and 4 % respectively ADH will thus induce relatively small changes in the mean transit times In order to check whether the large changes in mean transit time previously reported (Thurau et al 1960) could be due to induction of water diuresis with high amounts of fluid one nembutal anesthetized dog (No 9 in Table I) was infused with 1500 ml of the hypotonic solution before water diuresis was obtained As seen from the table all the red cell flow values were more or less unchanged as also were the total renal blood flow and the glomerular filtration rate

In Table I all data on the total and regional flows and the glomerular filtration rates are listed and represent the average value within each period As seen in the table the changes were fairly small and partly irregular making it difficult to draw any definite conclusions It seemed however that during water diuresis the total

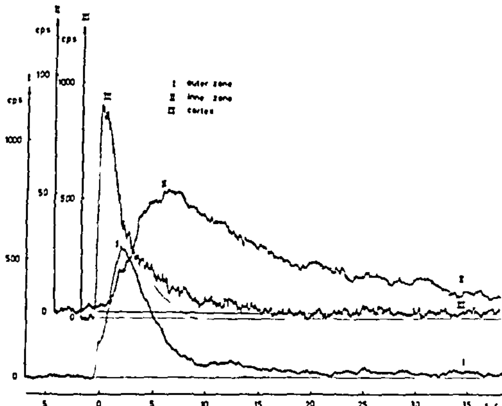


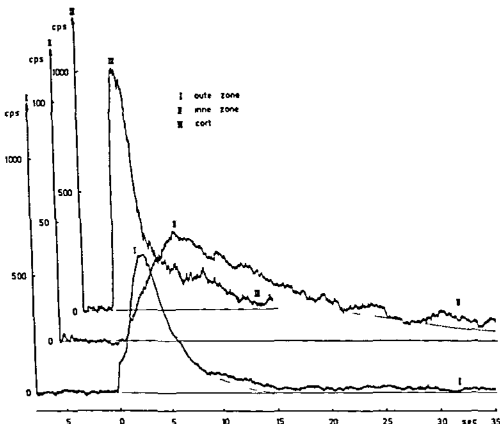

Fig 1 Cortical (III) outer medullary (I) and inner medullary (II) indicator-dilution curves obtained from experiment No 1 during water diuresis (left figure) and after infusion of arginine vasopressin at a rate of 1 5 mU/min and kg b wt (right figure)

renal blood flow and the glomerular filtration increased to some extent (see Table I and Fig 2) and were not restored after the ADH infusion In the same way the red cell flow increased in all the kidney region the estimated increases being 19 %, 18 % and 10 % for the cortical outer medullary and inner medullary circulation respectively The changes are however only just significant (Fig 3) After the infusion of ADH the red cell flow in the cortex and outer medulla (but not the inner medulla) *returned to the control values These changes are statistically significant* ($P < 0\,01$)

In the inner medulla which is the most interesting part in this connection the mean transit time decreased slightly on the average in water diuresis and increased again after ADH The red cell volume showed in consideration of the accuracy of this technique small and inconsistent changes but there might have been a true increase in red cell volume (14 %) during ADH induced antidiuresis

The results clearly indicate that neither water diuresis nor antidiuresis is accompanied by any major changes in the intrarenal blood flow conditions No important selective *effect on the inner medullary circulation* could thus be established

With the small changes reported it is obvious that these could easily be obscured




by spontaneous alterations in the blood flow conditions during the e relatively long experiments Thus it will take some 1—2 h to obtain water diuresis whereas ADH will have a more immediate effect The significant reduction in the red cell flow in the cortical and outer medullary parenchyma after ADH indicates therefore that there could have been a real increase in the red cell flow in water diuresis as well

## Discussion

The aim of this investigation was to determine the pure effect of water diuresis and also the effect of the species specific arginine vasopressin This meant that the effect of hydration usually concomitant with a water diuretic state should be minimized as well as the dehydration usually accompanying an antidiuretic state

The use of chloralose dissolved in saline meant by necessity that a large amount of fluid had to be administered and this was estimated at about 200 ml during the experiment The animals could then be considered well hydrated In order to avoid more serious effects of volume expansion water diuresis was induced by comparative

TABLE I Summary of circulatory parameters in the cortical outer medullary and inner medullary parenchyma during control antidiuresis water diuresis and antidiuresis as induced by the infusion of species specific arginin vasopressin The mean values calculated refers to experiment I–VIII

| Exp no | | CORTEX | | | OUTER MEDULLA | | |
|---|---|---|---|---|---|---|---|
| | | transit time sec | Red cell volume change | Red cell flow of control | transit time sec | Red cell volume change | Red cell flow of control |
| I | Control | [illegible] 5 | | 100 | 4 5 | | 100 |
| | $H_2O$ | [illegible] | +47 | 13[illegible] | 4 [illegible] | +9 | 10[illegible] |
| | ADH | [illegible] 8 | 30 | 100 | 5 1 | −7 | 90 |
| II | Control | [illegible] | | 100 | 4 0 | | 100 |
| | $H_2O$ | [illegible] 1 | +6 | 133 | [illegible] 5 | 1 | 158 |
| | ADH | 3.2 | +6 | 93 | 3 1 | +11 | 14[illegible] |
| III | Control | 3 0 | | 100 | 11 6 | | 100 |
| | $H_2O$ | [illegible] | +27 | 153 | 1[illegible] 6 | +30 | 1[illegible]0 |
| | ADH | 3 8 | +1[illegible] | 110 | 15 | +8 | 101 |
| IV | Control | [illegible] 5 | | 100 | 8 2 | | 100 |
| | $H_2O$ | 2 | [illegible] | 1[illegible]0 | 7 9 | −11 | 91 |
| | ADH | 2 [illegible] | 8 | 1[illegible]1 | 8 4 | +[illegible] | 88 |
| V | Control | [illegible] 4 | | 100 | – | – | – |
| | $H_2O$ | 2 | +4 | 170 | – | – | – |
| | ADH | [illegible] | 16 | 113 | – | – | |
| VI | Control | 2 4 | | 100 | 5 3 | | 100 |
| | $H_2O$ | 3 | +53 | 99 | 6 1 | −8 | 80 |
| | ADH | 3 4 | −51 | 2 | 7 5 | −4 | 6[illegible] |
| VII | Control | 5 | | 100 | – | – | – |
| | $H_2O$ | 2 [illegible] | 8 | 89 | – | – | |
| | ADH | 3 0 | 5 | 80 | – | – | – |
| VIII | Control | [illegible] 6 | | 100 | 4 3 | | 100 |
| | $H_2O$ | 2 4 | 0 | 104 | 3 9 | +78 | 150 |
| | ADH | 3 [illegible] | +1 | 80 | 4 [illegible] | +9 | 118 |
| IX | Control | [illegible] | | 100 | 6 8 | | 100 |
| | $H_2O$ | [illegible] | 9 | 91 | 6 1 | 13 | 97 |
| Mean | Control | [illegible]58 | | 100 | 6 31 | | 100 |
| | $H_2O$ | [illegible]61 | 19 | 119 | 6 27 | +8 | 118 |
| | ADH | 3 0 | 6 | 9[illegible] | 7 42 | 0 | 100 |

ly small amounts of a hypotonic (with respect to saline) solution The glucose added to the solution however caused an osmotic diuresis after the first single injection of 100 ml but had vanished at the time of the steady water diuretic state as judged by the absence of urinary glucose In any case it is obvious that the effects produced by the water diuresis are partly due to volume expansion and not solely to a decrease in plasma osmolality The ADH effects would in contrast probably have been due to this hormone alone since the degree of hydration remained essentially unchanged

The present findings are on the whole in agreement with those of Aukland (1966)

| INNER MEDULLA | | | RBF ml/min | GFR ml/min | Urine osmolality m osm/kg |
|---|---|---|---|---|---|
| transit time sec | Red cell volume change | Red cell flow of control | | | |
| 16 2 | | 100 | 290 | 39 8 | 596 |
| 15 3 | +25 | 132 | 262 | 39 1 | 180 |
| 19 9 | +16 | 114 | 270 | 41 6 | 462 |
| 13 2 | | 100 | — | 42 9 | 520 |
| 12 6 | 0 | 104 | — | 44 3 | 270 |
| 20 1 | +58 | 104 | — | 35 7 | 355 |
| 21 7 | | 100 | 210 | 26 5 | 617 |
| 18 3 | +16 | 137 | 233 | 28 5 | 130 |
| 30 9 | +23 | 98 | — | — | 500 |
| 25 9 | | 100 | 185 | 41 1 | 896 |
| 20 2 | −10 | 106 | 231 | 45 0 | 208 |
| 22 0 | +15 | 123 | 220 | 54 9 | 612 |
| 7 1 | | 100 | 190 | 30 0 | 981 |
| 6 9 | −9 | 94 | 275 | 38 1 | 279 |
| 7 4 | −9 | 82 | 268 | 43 4 | 534 |
| 18 7 | | 100 | — | 23 3 | 1087 |
| 19 2 | +7 | 104 | 190 | 27 3 | 244 |
| 21 4 | +14 | 106 | 200 | 30 8 | 545 |
| 19 8 | | 100 | 190 | 20 6 | 330 |
| 18 8 | +2 | 107 | 254 | 23 7 | 141 |
| 15 8 | +11 | 141 | — | 23 9 | 338 |
| 18 0 | | 100 | 333 | 32 0 | 579 |
| 21 2 | +10 | 94 | 411 | 40 7 | 166 |
| 18 2 | −13 | 96 | 380 | 36 3 | 756 |
| 20 3 | | 100 | 392 | 31 5 | 683 |
| 20 6 | +7 | 106 | 417 | 32 5 | 250 |
| 17 58 | | 110 | 233 0 | 32 8 | 701 |
| 16 56 | +5 | 108 | 251 0 | 36 9 | 202 |
| 19 46 | +14 | | 268 0 | 38 1 | 513 |

who reported that ADH had no effect on the outer medullary blood flow as judged from the wash out rate of hydrogen internally detected by platinum electrodes Thurau *et al* (1960) using a dye dilution technique with detection with small photocells placed along the pelvic surface of the medulla found shortening of the mean transit time during water diuresis (to about 50 % of control) and prolongation after administration of ADH

With an unchanged plasma volume the result would imply an (approximately 2 fold) increase in the inner medullary blood flow in water diuresis *On direct*

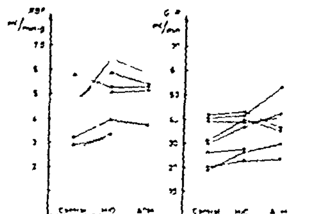


Fig. 2. Total renal blood flow (RBF) and glomerular filtration rate (GFR) during the control antidiuretic state, after the induction of water diuresis and during antidiuresis as evoked by infusion of arginine vasopressin.

puncture of vasa recta the protein concentration has been found to be twice as high in antidiuresis as in mannitol diuresis (Thurau *et al.* 1963, Wilde *et al.* 1963). This would then mean that the "volume of distribution" of plasma should be higher in antidiuresis than in conditions with depletion of the medullary osmotic gradient. The results of Thurau would then be compatible with a more or less unchanged inner medullary blood flow. The finding of only slight changes in the red cell volume in the present investigation is, however, in a way contradictory to this argument.

In addition to more or less direct measurements of the medullary blood flow as above, indirect evidence of an action of ADH has been reported by several authors. Thus Fourman and Kennedy found reduced staining of a fluorescent dye in the

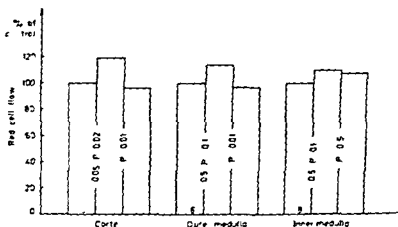



Fig. 3. Relative cortical, outer medullary and inner medullary red cell flow during the control antidiuretic condition, after the induction of water diuresis and during antidiuresis induced by Arginine vasopressin. The figure shows that water diuresis is concomitant with a small and barely significant increase in the red cell flow in all the regions studied. Arginine vasopressin causes a reduction towards the control values except for the inner medulla where the red cell flow remains essentially unchanged.

medullary vessels in antidiuresis which was interpreted as a reduced blood flow Wunderlich and Schnermann (1969) on measuring the hydrostatic pressure in vasa recta reported a reduction in pressure from 13 to 8 mm Hg when ADH in subpressor doses was given to rats with diabetes insipidus

In conclusion it would seem that a change-over from antidiuresis to water diuresis in the dog by means of administration of reasonable amounts of fluid is accompanied by only small and probably insignificant changes in the renal medullary blood flow ADH in physiological doses has only minor effects on the renal circulation and no specific action on the juxtamedullary circulation was established in this study These arguments do not however exclude the possibility that the hydration and dehydration *per se* could be accompanied by changes in renal medullary blood flow as found by Harsin and Pelley (1965) using the 86 Rb accumulation technique

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# Stimulation of Lymphocyte Release from the Spleen by Theophylline and Isoproterenol

By

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## Abstract

Ernström U and G Sandberg *Stimulation of lymphocyte release from the spleen by theophylline and isoproterenol* Acta physiol scand 1974 90 202—209

The effect of theophylline and isoproterenol on the splenic release of lymphocytes into the blood was studied in guinea pigs by the comparison between the content of cells in splenic afferent and efferent blood combined with determination of the blood flow through the spleen

An intracardial injection of theophylline like that of isoproterenol resulted in a greatly increased release of lymphocytes from the spleen as compared to that in control animals. The effect of theophylline in contrast to that of isoproterenol could not be abolished by pretreatment with propranolol The similar effects of theophylline (known to inhibit the degradation of cAMP) and isoproterenol (known to stimulate the synthesis of cAMP) on the splenic release of lymphocytes to the blood suggest that these effects may be mediated by an increase of intracellular cAMP *This suggestion is supported by our finding of an additive effect when* theophylline and isoproterenol were administered in combination

The abovementioned release of lymphocytes was found shortly after administration of the drug After isoproterenol it was followed by an inhibited release of lymphocytes from the spleen This was in contrast to the much longer duration of the releasing effect of theophylline

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Blood from the splenic vein contains more lymphocytes than that from the splenic artery at least in guinea pigs (Ernstrom and Sandberg 1968 Sandberg 1972 a) and rat Sandberg unpublished results) The spleen is therefore considered to produce lymphocytes and to release such cells into the blood (Ernstrom and Sandberg 1972 Sandberg 1972 a)

In a previous investigation we studied the effects of adrenergic $\alpha$ and $\beta$ receptor stimulating drugs on the release of lymphocytes and granulocytes from the spleen (Ernstrom and Sandberg 1973) Administration of noradrenaline (NA) or isoproterenol considerably increased the number of lymphocytes and granulocytes in the splenic venous blood within a few min which was interpreted as a mobilization of white cells preformed and stored in the spleen By aid of blocking drugs evidence was obtained that the effects of NA were mediated by stimulation of adrenergic $\alpha$ receptors and the effects of isoproterenol by $\beta$ receptors

Many effects of $\beta$ receptor stimulation are mediated by cAMP (Robison *et al* 1967) It is also known that in lymphocytes cultured *in vitro* the amount of cAMP

increases after incubation with isoproterenol (Smith *et al* 1969 Bourne and Melmon 1971 Henney *et al* 1972) The purpose of the present paper was to study the effects of theophylline on the splenic release of cells in order to test the possibility that isoproterenol exerts its effect by increasing the level of cAMP Theophylline interferes with the degradation of cAMP by the inhibition of the phosphodiesterase activity and thus increases the intracellular concentration of cAMP without the stimulation of $\beta$ receptors (Butcher and Sutherland 1969)

## Material and methods

Male guinea pigs weighing 250—300 g were used for the experiments Different groups of animals were treated according to the following schedule

(1) Guinea pigs were given a single injection of theophylline (ACO Stockholm Sweden) intracardially in a volume of 0 2 ml containing a dose corresponding to 60 mg per kg b wt Blood samples from the animals were taken after 5 min 20 min 2 h and 24 h Control animals were given saline intracardially and investigated after 5 min

(2) Guinea pigs were given daily injections of theophylline as above during 4 consecutive days and blood samples were taken 5 min and 24 h after the last injection Control animals were instead given daily injections of saline and otherwise treated the same way

(3) Guinea pigs were pretreated with propranolol (ICI England) intraperitoneally in a dose of 8 mg per kg Theophylline as above was given after 2 h and the animals were examined 5 min later

(4) Guinea pigs were given a single injection of isoproterenol (AB Hassle Goteborg Sweden) intracardially in a volume of 0 05 ml containing a dose corresponding to 150 $\mu g$ per kg b wt Different groups of animals were investigated after 5 min 30 min 2 h 24 h and 48 h. Control animals were given saline intracardially and investigated after 2 h

(5) Guinea pigs were treated with both theophylline and isoproterenol in an attempt to obtain an additive effect of the two drugs In a first experiment the two drugs were given in doses as above theophylline 2 h and isoproterenol 5 min before investigation In a second attempt the doses were lowered (theophylline 30 mg per kg isoproterenol 90 $\mu g$ per kg) and the two drugs were given 5 min before investigation each alone as well as in combination

The number of animals in each experiment is presented in Table I II III and IV

*Blood sampling* The guinea pigs were anesthetized with 2 5 per cent pentobarbital sodium (25—50 mg per kg intraperitoneally) The peritoneal cavity was incised over the spleen After incision into the splenic vein 25 $\mu l$ of blood was collected in a heparinized pipette Immediately afterwards 25 $\mu l$ of blood from the splenic artery was withdrawn by the same procedure The blood samples were used for white cell counts in a Burker chamber Mono- and polynuclear cells were counted separately Blood was also collected in heparinized capillary tubes for determination of the hematocrit by centrifugation for 5 min at 9000 $g$

*Preparation of the spleen* The spleen was excised weighed and divided into two parts One part was used for determination of the total number of nucleated cells in the spleen The specimen was cooled in a buffered phosphate solution with pH 7 25 cut into small pieces and then gently pressed with a glass piston over a nylon net while being rinsed with the cold buffer The suspension of cells obtained was passed through nylon nets with decreasing mesh size using slight suction in order to dissociate the cells The number of mononuclear cells in the suspension was determined by counting in a Burker chamber and the total number of mononuclear cells in the spleen was calculated

The other part of the spleen was prepared for histology by fixation in formaldehyde embedding in paraffin wax and sectioning The sections were stained with Ladewig

*Blood flow measurements* By use of the xenon 133 clearance method the splenic blood flow was measured in guinea pigs treated with theophylline (60 mg per kg) or saline The measurement were started within 1 min after the treatment with the drug and performed over a period of 10 min which covers the time when blood samples were taken for white cell counts (made in a separate group of animals) For details on the technique see Sandberg 1972 b

*Statistical analysis* The veno-arterial differences obtained from the individual animals were used for calculation of the mean difference and standard error of the mean For testing of statistical significance Student's t test was used The release of cells per min was calculated from the mean veno-arterial difference and the mean blood flow determined independently in separate groups of animals The standard error of the product was calculated by use of the formula (Dahlberg 1940)

TABLE I. Effect of theophylline (60 mg per kg) on the content of lymphocytes and granulocytes in splenic venous and arterial blood. Mean ± S.E.

| Treatment | Interval between treatment and investigation | Number of animals | Splenic veno-arterial difference: Lymphocytes per µl | Splenic veno-arterial difference: Granulocytes per µl | Splenic arterial blood: Lymphocytes per µl | Splenic arterial blood: Granulocytes per µl |
|---|---|---|---|---|---|---|
| Saline | 5 min | 15 | [illegible]34 ** ± 103 | 17 * ± 66 | 157[illegible] ± 161 | 1318 ± 18[illegible] |
| Theophylline | 5 min | 17 | [illegible]623 ** ± 338 | 597 ± 373 | 277[illegible] ± 345 | 3554 ± 615 |
| Theophylline | 20 min | 9 | 1911 ± 718 | 249 ± 291 | 2037 ± 278 | 1609 ± [illegible] |
| Theophylline | 2 h | 9 | 35[illegible]7 ± 968 | 876 ± 537 | 2359 ± 203 | 3[illegible]64 ± 533 |
| Theophylline | 24 h | 1[illegible] | 1550 * ± 363 | 232 ± 291 | 1890 ± 130 | 1934 ± 381 |
| Theophylline and Propranolol | 5 min, 2 h | 19 | [illegible]71*** ± 399 | 432 ± 374 | 2764 ± 131 | 2036 ± 745 |

*, **, *** indicates the existence of a statistically significant veno-arterial difference (p < 0.05, p < 0.01 and p < 0.001 respectively).

$$S_{[illegible]} = \pm\sqrt{\bar{x}\,(S_{[illegible]})^2 \ [illegible]\ \bar{y}\,(S_{[illegible]})}$$

Where $\bar{x}$ = mean blood flow, $S_{\bar{x}}$ = standard error of $\bar{x}$ (S.E.), $\bar{y}$ = mean veno-arterial difference, $S_{\bar{y}}$ = standard error of $\bar{y}$.

## Results

*Effect of theophylline on splenic blood flow*

The injection of theophylline slightly reduced the splenic blood flow per g tissue but not to a statistically significant extent (Table V). The effect was similar to that of isoproterenol (Ernström and Sandberg 1973). Due to a higher splenic weight the total blood flow was almost identical in the theophylline treated and in the saline treated animals. As the blood flow was not significantly influenced, a major change of the splenic veno-arterial difference in number of lymphocytes or granulocytes after treatment with theophylline indicates an altered uptake or release of cells in the spleen.

*Effect of a single injection of theophylline*

Theophylline (60 mg per kg) markedly increased the difference in content of lymphocytes between splenic venous and arterial blood as compared to control (Table I). This increase was evident already within 5 min after the injection ($p < 0.001$) and persisted after 20 min, 2 h and 24 h ($p < 0.01$, $p < 0.05$ and $p < 0.01$). As regards the granulocytes, no significant increase of the veno-arterial difference was found. The content of both lymphocytes and granulocytes in splenic arterial blood was increased after the administration of theophylline ($p < 0.01$), the highest value found after 5 min (Table I). There was no difference in the hematocrit between splenic afferent and efferent blood in any of the experimental groups treated with theophylline and there was no change in the hematocrit of the blood.

TABLE II Effect of 4 daily injections of theophylline (60 mg per kg) on the content of lymphocytes and granulocytes in splenic venous and arterial blood Mean ± S E

| Treatment | Interval between treatment and investigation | Number of animals | Weight of spleen mg | Splenic veno-arterial difference | | Splenic arterial blood | |
|---|---|---|---|---|---|---|---|
| | | | | Lymphocytes per µl | Granulocytes per µl | Lymphocytes per µl | Granulocytes per µl |
| Saline | 5 min | 10 | 421 ± 15 | 1052 * ± 271 | 436 ± 350 | 2076 ± 178 | 1993 ± 768 |
| Theophylline | 5 min | 11 | 365 ± 23 | 2380*** ± 289 | 891 ** ± 172 | 1644 ± 88 | 1431 ± 167 |
| | | | N S | p < 0 05 | N S | N S | N S |
| Saline | 24 h | 7 | 461 ± 15 | 1180 ** ± 200 | 187 ± 130 | 2638 ± 246 | 1105 ± 191 |
| Theophylline | 24 h | 8 | 385 ± 25 | 833** ± 234 | 236 ± 117 | 2665 ± 301 | 1299 ± 188 |
| | | | p < 0 05 | N S | N S | N S | N S |

* * indicate the existence of a statistically significant veno arterial difference ($p < 0.01$ and $p < 0.001$ respectively) The p-values in the table indicate statistically significant effect of the treatment with theophylline N S = not significant

*Effect of repeated injections of theophylline*

When the animals were given repeated injections with theophylline (60 mg per kg) during 4 consecutive days and examined immediately after the last injection an increased veno arterial difference in content of lymphocytes could still be demonstrated ($p < 0.05$) in comparison with saline treated controls (Table II) Also a statistically significant difference in content of granulocytes was now found The arterial blood leucocyte content was low The weight of the spleen was decreased in the theophylline treated animals (Table II) and the number of mononuclear spleen cells was reduced from $103 \pm 8 \times 10^6$ in saline treated animals to $87 \pm 8 \times 10^6$ A histological examination of splenic sections did not disclose any obvious difference between animals treated with theophylline and with saline

When examined 24 h after the last of 4 daily injections of theophylline the splenic veno arterial difference in content of lymphocytes and granulocytes did not differ from that in saline treated controls (Table II) neither did the number of lymphocytes or granulocytes in the splenic arterial blood

*Effect of a single injection of theophylline after pretreatment with propranolol*

Propranolol administered at a time and in a dose which in a previous investigation completely inhibited the effect of isoproterenol on the mobilization of leucocytes from the spleen (Ernstrom and Sandberg 1973) failed to inhibit the lymphocyte releasing effect of theophylline (Table I) Thus the release of lymphocytes in the theophylline injected animals pretreated with propranolol was significantly greater than in the saline treated ($p < 0.01$) and not different from that in the animals only injected with theophylline

*Effect of a single injection of isoproterenol*

Isoproterenol (150 µg per kg) markedly increased the difference in content of lymphocytes between splenic venous and arterial blood within 5 min as reported earlier (Ernstrom and Sandberg 1973) After 30 min 2 h and 24 h no such veno

TABLE III. Effect of isoproterenol (150 μg per kg) on the content of lymphocytes and granulocytes in splenic venous and arterial blood. Mean ± S.E.

| Treatment | Interval between treatment and investigation | Number of animals | Splenic veno-arterial difference | | Splenic arterial blood | |
|---|---|---|---|---|---|---|
| | | | Lymphocytes per μl | Granulocytes per μl | Lymphocytes per μl | Granulocytes per μl |
| Saline | 2 h | 11 | 852 ±287 | 442 ±2[illegible]4 | 2536±196 | 3125±576 |
| Isoproterenol | 5 min | 14 | 3047** ±848 | 1735* ±359 | 2104±318 | 1398±269 |
| Isoproterenol | 30 min | 8 | 4 ±180 | −379* ±162 | 1746±217 | 2247±8[illegible]4 |
| Isoproterenol | 2 h | 13 | −110 ±273 | −137 ±531 | 2482±183 | 5473±931 |
| Isoproterenol | 24 h | 10 | 256 ±196 | −318 ±208 | 2008±205 | 1850±273 |
| Isoproterenol | 48 h | 9 | 1364* ±309 | 1037* ±466 | 2046±240 | 2472±410 |

*, **, *** indicate the existence of a statistically significant veno-arterial difference (p < 0.05, p < 0.01 and p < 0.001 respectively).

arterial difference was obtained. Thus the difference found in normal or saline treated animals was abolished. After 48 h a significant difference was again obtained, slightly higher than that in saline or untreated animals. Similarly the splenic veno-arterial difference in the number of granulocytes was increased 5 min after isoproterenol, then abolished after 30 min, 2 h and 24 h and returned after 48 h. The content of white cells in splenic arterial blood was not significantly changed by the injection of isoproterenol (Table III). Microscopically the spleen of animals treated with isoproterenol was not depleted in cells.

*Effect of combined treatment with theophylline and isoproterenol*

Combined treatment with theophylline (60 mg per kg 2 h before investigation and

TABLE IV. Effect of theophylline and isoproterenol alone or in combination on the content of lymphocytes and granulocytes in splenic venous and arterial blood. Mean ± S.E.

| Treatment | Interval between treatment and investigation | Number of animals | Splenic veno-arterial difference | | Splenic arterial blood | |
|---|---|---|---|---|---|---|
| | | | Lymphocytes per μl | Granulocytes per μl | Lymphocytes per μl | Granulocytes per μl |
| Theophylline (30 mg/kg) | 5 min | 9 | 2480** ±636 | 1473* ±615 | 1930±188 | 2448±276 |
| Isoproterenol (90 μg/kg) | 5 min | 9 | 2156*** ±370 | 1632** ±348 | 2123±353 | 2270±819 |
| Theophylline (30 mg/kg) and isoproterenol (90 μg/kg) | 5 min; 5 min | 9 | 5066** ±1368 | 5501* ±2343 | 1961±188 | 2468±919 |
| Theophylline (60 mg/kg) and isoproterenol (150 μg/kg) | 2 h; 5 min | 11 | 3516*** ±545 | 1111 ±573 | 2160±297 | 2482±437 |

*, **, *** indicate the existence of a statistically significant veno-arterial difference (p < 0.05, p < 0.01 and p < 0.001 respectively).

isoproterenol (150 $\mu g$ per kg 5 min before investigation) resulted in a splenic veno-arterial difference in content of lymphocytes comparable to that seen after each drug alone. Thus no additive effect was obtained in this experiment (Table IV).

On the other hand an additive effect was obtained when the doses of theophylline and isoproterenol were reduced to 30 mg and 90 $\mu g$ respectively and both drugs were given simultaneously 5 min before investigation (Table IV). Due to a variance higher than expected the number of animals used was just not sufficient for obtaining a statistically significant additive effect when using Student's $t$ test. However with two non-parametric tests (Mann Whitney test and Tukey's Quick test, see Conover 1971) the splenic veno-arterial difference after both drugs was found to be significantly higher than after each drug alone ($p < 0.05$ with both tests).

*Effect of theophylline on the splenic release of lymphocytes per min*

As mentioned above the treatment with theophylline did not significantly change the splenic blood flow. This indicates that the observed increase in splenic veno-arterial difference in number of lymphocytes after treatment with theophylline reflects an accelerated output of such cells from the spleen into the blood. The number of lymphocytes and granulocytes leaving the spleen per min are calculated in Table V. For comparison the results obtained with isoproterenol are included in the table (from Ernström and Sandberg 1973). It can be seen that both theophylline and isoproterenol significantly increased the release of lymphocytes from the spleen in comparison with saline treated animals. As regards the granulocytes a significant release was obtained with isoproterenol but not with theophylline.

## Discussion

The administration of theophylline was found to increase the difference between the number of lymphocytes per $\mu l$ of splenic efferent and afferent blood considerably. The splenic blood flow in $\mu l$ per min was not significantly changed. A calculation of the splenic release of lymphocytes per min showed an increase from $1.4 \pm 0.3 \times 10$ in normal animals to $5.2 \pm 1.2 \times 10$ ɔ min after theophylline (60 mg per kg). Thus theophylline in the dose used increased the splenic release of lymphocytes into the blood to about the same extent as isoproterenol which increased the release to $7.4 \pm 2.0 \times 10$

The effect of theophylline could be registered very early after its administration indicating that the primary effect must be a mobilization of preformed cells. Surprisingly the output of splenic lymphocytes was still high as late as 24 h after the administration of theophylline. An increased release of lymphocytes for an extended period of time without a compensatory proliferation of cells should result in cellular depletion of the spleen. We therefore investigated the effect of 4 daily injections of theophylline, a treatment which should deplete the spleen severely in the absence of an increased proliferation. The treatment reduced the splenic weight and content of mononuclear cells only slightly and not to the expected extent. On the 4th day theophylline was still able to mobilize cells from the spleen within 5 min although

TABLE 3. Calculation of the release of lymphocytes and granulocytes from the spleen after treat

| Treatment | Splenic weight | Splenic veno-arterial difference | |
|---|---|---|---|
| | | Lymphocytes per μl | Granulocytes per μl |
| Saline | 380 ± 25 | 734 ± 103 (15) | 172 ± 66 |
| Theophylline | 443 ± 23 | 2673 ± 358 (17) | 597 ± 393 |
| | | $p < 0.001$ | N.S. |
| Isoproterenol | 507 ± 74 | 3647 ± 848 (14) | 1935 ± 359 |
| | | $p < 0.01$ | $p < 0.01$ |

The p-values indicate a statistically significant increase of the splenic veno-arterial difference in number of cells and of the splenic release of cells per min after treatment with theophylline or isoproterenol as compared to the results in the saline treated controls. N.S. = not significant. The results with isoproterenol are taken from Ernström and Sandberg (1973) and are included in the table for comparison. Number of animals within parenthesis.

this time the effect was abolished 24 h later apparently due to the daily treatment with theophylline. It is possible that theophylline in addition to an immediate mobilizing effect stimulates the proliferation of splenic cells. This would explain the prolonged effect and the lack of a severe depletion of cells in the spleen. Theophylline is known to inhibit the enzymatic degradation of cAMP and in fact a stimulatory effect of cAMP on cellular proliferation has been demonstrated *in vitro* for thymic lymphocytes and hemopoietic stem cells (MacManus and Whitfield 1969, Byron 1971).

Isoproterenol like theophylline increases the release of splenic lymphocytes within 5 min after administration. This effect of isoproterenol in contrast to that of theophylline can be blocked by pretreatment with propranolol (Ernstrom and Sandberg 1973) and thus seems to be mediated by stimulation of β receptors. We suggest that *the early effects of both isoproterenol and theophylline on the splenic release of cell* to the blood are mediated by cAMP in the same effector cells. In order to obtain experimental evidence supporting this hypothesis both drugs were given to the same animals and a possible additive effect was looked for. In high doses no addition was obtained probably indicating that maximal stimulation had been obtained with each drug alone. When doses of isoproterenol and theophylline with submaximal effect were administered simultaneously shortly before investigation a distinct additive effect on the release of lymphocytes as well as granulocytes from the spleen was demonstrated. Our results indicate that isoproterenol and theophylline via cAMP causes an increased release of white blood cells from the spleen. In the present paper we have not tried to decide whether the effector cells are the leucocytes themselves, smooth muscle cells or some other cell type in the spleen. One possibility is that the migratory properties of the lymphocytes and granulocytes are increased by cAMP.

In contrast to the prolonged release of lymphocytes from the spleen caused by theophylline the initial mobilization of cells brought about by isoproterenol was fol

ment with theophylline Mean ± S E

| Splenic blood flow | | Splenic release | |
|---|---|---|---|
| μl/g min | μl/min | Lymphocytes × 10⁻ per min | Granulocytes × 10⁻⁶ per min |
| 513 8 ± 74 3 (11) | 195 4 ± 31 0 | 1 4 ± 0 3 | 0 3 ± 0 1 |
| 444 5 ± 79 2 (9) | 197 1 ± 36 6 | 5 2 ± 1 2 | 1 2 ± 0 7 |
| N S | N S | p < 0 01 | N S |
| 403 2 ± 54 0 (11) | 204 3 ± 29 0 | 7 4 ± 2 0 | 2 5 ± 0 8 |
| N S | N S | p < 0 01 | p < 0 05 |

lowed by a depressed release of both lymphocytes and granulocytes This difference in effect between the two drugs can be explained by the assumption that theophylline has a dual effect In addition to the early mobilizing effect on splenic cells theophylline may also stimulate the *proliferation of splenic lymphocytes* as suggested above

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Acta physiol scand 1974 90 210—217

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# Muscle Glycogen Utilization during Exercise after Physical Training

By

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## Abstract

KARLSSON J L O NORDESJO and B SALTIN *Muscle glycogen utilization during exercise after physical training* Acta physiol scand 1974 90 210—217

, male subjects exercised at a mean oxygen uptake of 2 3 $l \times min^{-1}$ ($\approx 65$ of $\dot{V}_{O_2}$ max) before and after 2 months of physical training for 90 min and 120 min respectively Mean maximal oxygen uptake increased by 11 ($p < 0.05$) and the mean R value during the submaximal work decreased from 0 93 to 0 89 ($p < 0.01$) Plasma levels for FFA and glucose during exercise were unaffected by training The mean rate of muscle glycogen breakdown in the thigh between 0 and 90 min of prolonged work could be estimated to be 0 66 before and 0 41 mmol glucose units $\times kg^{-1}$ wet muscle $\times min^{-1}$ after training ($p < 0.01$) Part of the reduced glycogen utilization could be explained by a less pronounced lactate production in the trained stage It is concluded that a short period of physical conditioning results in a decrease in glycogen utilization and an enhanced fat oxidation


When subjects with different fitness levels exercise for a more prolonged period of time at the same absolute work intensity a lower respiratory exchange ratio (R) is found in the most well trained subjects (Christensen and Hansen 1939 indicating a less pronounced carbohydrate utilization during work in subjects with a high physical work capacity

Furthermore it has in cross sectional studies been found that subjects with different physical work capacity had similar rates of glycogen depletion when working at the same relative work load for a prolonged period of time (Hermansen Hultman and Saltin 1967 It has also been found that with increase in the relative work load the glycolytic rate increases (Saltin and Karlsson 1971)

The question then arises as to what extent a short period of physical training leading to an increase in maximal oxygen uptake can produce a change in the metabolic response to prolonged exercise resulting in a glycogen sparing effect

The aim of the present study was therefore to investigate the muscle glycogen breakdown in the thigh during prolonged work in previously untrained men before and after a couple of months of physical conditioning

HEART RATE, beats min⁻¹
before training
after "
<0.05 <0.001 <0.05 <0.01 P
TIME, min.

OXYGEN UPTAKE l min⁻¹
TIME min.

RESPIRATORY EXCHANGE RATIO
<0.05 <0.05 P
TIME, min.

MUSCLE GLYCOGEN
mmol glucose units kg⁻¹ wet muscle
P < 0.05
TIME, min.

Fig 1 Mean values for different variables in 5 subjects at rest and during work tests at the same absolute work load (mean oxygen uptake 2 3 l × min⁻¹) before and after 8 weeks of interval training The significance of mean differences at equivalent points of time and between values at 90 and 120 min has been tested by the t test and the degree of significance has been included in the figure

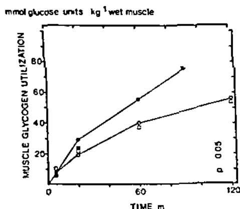



Fig 2 The relationship between the utilization of muscle glycogen and work time before (●) and after (○) training The significance of mean differences has been tested as in Fig 1 Mean values for the 3 subjects who had the same initial content of muscle glycogen before (■) and after (□) training have also been included in the figure

TABLE I. Values at rest and during prolonged work at a mean load of [illegible] W

| Variable | | Rest | | Prolonged work, min | |
|---|---|---|---|---|---|
| | | | | [illegible] | |
| | | b | a | b | a |
| Heart rate beats/min | $\bar{x}$ | | | 140.0 | 139.0 |
| | SD | | | 13.3 | 14.0 |
| Oxygen uptake l/min | $\bar{x}$ | | | | |
| | SD | | | | |
| R | $\bar{x}$ | | | | |
| | SD | | | | |
| Muscle glycogen mmol glucose units/kg w.m. | $\bar{x}$ | 84.4 | 65.4 | 7.2 | [illegible] |
| | SD | 16.6 | 5.2 | 14.5 | [illegible]0 |
| Muscle lactate mmol/kg w.m. | $\bar{x}$ | 0.88 | 0.86 | 8.80 | 6.6 |
| | SD | 0.15 | 0.09 | 3.37 | 3.5 |
| Blood lactate mmol/l blood | $\bar{x}$ | 1.48 | 1.26 | 5.1 | 4.09 |
| | SD | 0.27 | 0.17 | 1.58 | 1 |
| Blood glucose mmol/l blood | $\bar{x}$ | 3.97 | 4.09 | | |
| | SD | 0.57 | 0.[illegible] | | |
| FFA in plasma mmol/l | $\bar{x}$ | 0.53 | 0.[illegible]4 | | |
| | SD | 0.09 | 0.11 | | |

b = before training
a = after 2 months interval training
c after 2 months training heart rate at 90 min was 159.4 (SD 14.5) beats/min
d value at 15 min. After 30 min work the values were 4.40 (b) and 3.34 (a)

## Subjects

5 conscripts volunteered to participate in the study. Prior to the study they were fully informed about the procedure to be used. At induction the subjects were on an average [illegible] years old (range 19—2[illegible] years) with a mean height of 1[illegible]9 cm (range 1[illegible]3—184 cm) and a mean weight of 74.0 kg (range [illegible]—8[illegible] kg) which is 7.6 kg more ($p < 0.05$) than for a random sample of 18-year-old boys (Ahlborg *et al.* 19[illegible]). Maximal oxygen uptake before training averaged 3.[illegible] l · min⁻¹ (48 ml × kg⁻¹ · min⁻¹) which is 9 [illegible] higher than the mean value for two groups of conscripts of the same age (unpublished data). The maximal work load that could be sustained for 6 min was [illegible]0 watt which is 14 [illegible] higher than for conscripts at registration (Linroth 1969).

All subjects had physically very light occupations prior to induction. One of the subjects was physically inactive in his spare time. The remaining 4 subjects were somewhat active (grade [illegible] on a 4-graded score, Saltin and Grimby 1968).

## *Methods and Procedure*

The subjects underwent a series of preliminary tests including a short submaximal work test to habituate the subjects.

A few days later a graded submaximal work test was performed. After half an hour of rest the subjects performed maximally on a constant load. This procedure was repeated two days later. On the basis of the maximal tests a work load which could easily be sustained for 90 min was calculated and used during a prolonged exercise test 5 days later. The same procedure and identical work load were repeated after two months of training with the difference that the prolonged exercise now continued for 1[illegible]0 min and with some variation in the number of days between maximal and prolonged work tests. This variation is of negligible importance for the results.

| 20 | | 60 | | 90 | 120 |
|---|---|---|---|---|---|
| b | a | b | a | b | a |
| 163 8 | 147 8 | 171 6 | 155 4 | 178 0[c] | 161 4 |
| 15 4 | 19 3 | 18 7 | 9 7 | 15 9 | 14 4 |
| 2 33 | 2 32 | | | 2 57 | 2 48 |
| 0 24 | 0 26 | | | 0 23 | 0 27 |
| 0 95 | 0 91 | | | 0 90 | 0 87 |
| 0 05 | 0 04 | | | 0 05 | 0 05 |
| 55 2 | 45 8 | 28 7 | 25 0 | 8 8 | 9 8 |
| 10 7 | 3 5 | 10 3 | 10 3 | 4 0 | 7 1 |
| 7 56 | 5 94 | 6 12 | 1 84 | 4 76 | 1 52 |
| 3 62 | 4 56 | 2 57 | 0 75 | 2 61 | 0 73 |
| 5 06[a] | 3 70[a] | 3 34 | 2 52 | 3 12 | 2 02 |
| 1 40 | 1 39 | 0 83 | 0 70 | 0 61 | 0 47 |
| 3 46 | 3 87 | 3 80 | 3 54 | 3 84 | 3 23 |
| 0 73 | 0 37 | 0 92 | 0 43 | 1 02 | 0 29 |
| 0 51 | 0 41 | *0 73* | *0 72* | *1 01* | *1 00* |
| 0 14 | 0 06 | 0 21 | 0 20 | 0 28 | 0 18 |

During the training period the subjects underwent 21 sessions of physical conditioning which was characterized by repeated short (15 s) *maximal or close to maximal runs* with intervening periods (15 s) of jogging or walking ("interval training") The total running time at maximum was 6 h A detailed description of the training is given elsewhere (Knuttgen *et al* 1973)

During the work *tests ECG was recorded on a Elema Mingograf 34* and the *heart* rate was calculated from 20 R—R intervals Expired air was collected in Douglas bags from the 4th min onwards in the maximal work tests and after 20 and 90 (or 120) min of *exercise during* the prolonged work test. Gas analyses were performed by the Haldane technique The highest $\dot{V}_{O_2}$ before and after training are both mean values of two trials Capillary samples of blood were taken after the maximal and during the prolonged work tests and the lactate content was determined by an enzymatic method (Scholz *et al* 1959)

On the *day of the prolonged work test the subjects came to* the laboratory after a light breakfast. In conjunction with the prolonged work test samples of venous blood were taken at rest and after 20 60 and 90 (or 120) min of exercise for the determination of glucose (Hjelm and de Verdier 1963) and FFA (Trout Estes and Friedberg 1960)

During the prolonged work test muscle biopsies were taken with the Bergstrom technique (1962) from the lateral portion of the quadriceps muscle at rest and after 5 20 90 (or 120) min of work The samples of muscle tissue were analyzed for glycogen glucose and lactate according to *technique described by Karlsson (1971) The water content of the samples* varied from 76 at rest to 77 and 78 % during exercise respectively

The submaximal and the maximal work tests were performed on a calibrated electrically braked bicycle ergometer (Elema Schonander model AM 369) and the prolonged work test on an older model (AM 361 Holmgren and Mattson 1954) During the work tests the room temperature varied between 21 and 24 C

Conventional statistical methods were used and the results of training were evaluated by *means of a common t test applied to the mean of differences between paired observations* (Snedecor and Cochran 1967)

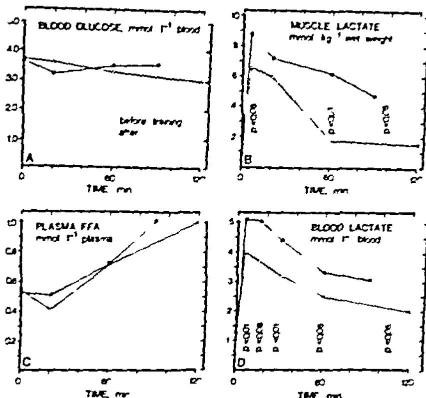



Fig. 3 See legend for Fig. 1

## Results

Maximal oxygen uptake increased in all subjects as an effect of the physical training. The mean improvement was from 3.56 to 3.95 l/min⁻¹ or 11 % ($p < 0.05$). Table I gives the values at rest and during the prolonged work test for the studied variables. During the prolonged work period significantly lower heart rates were observed after training as compared to before training (Fig. 1). Both before and after training the oxygen uptake during the prolonged work test (Fig. 1) was about 2.3 l · min⁻¹ after 20 min of work and slightly higher ($p < 0.01$) at the end of the exercise (90 or 120 min).

Before training the R values averaged 0.95 after 20 min of work and 0.90 at the end. After training the corresponding values were 0.91 and 0.87, respectively (Fig. 1). The differences at 20 min and at the end of work were significant at the 0.05 level.

After training muscle glycogen was significantly lower at rest ($p < 0.05$) and after 5 min of work ($p < 0.05$). During the remainder of the work the values of muscle glycogen were not significantly different before and after training (Fig. 1). The amount of muscle glycogen broken down during the first 5 min of exercise was very similar before and after training (Fig. 2). From then on its appears as if less glycogen has been utilized after physical conditioning and at the end of work a significantly ($p < 0.05$) lower amount of glycogen has been broken down.

The concentration of lactate in muscle and blood averaged at rest about 0.9 $mmol \times kg^{-1}$ wet muscle and 1.4 $mmol \times l^{-1}$ blood both before and after training. At onset of exercise there was a marked increase in lactate concentration reaching 8.8 $mmol \times kg^{-1}$ wet muscle (Fig. 3) and 5.1 $mmol \times l^{-1}$ blood (Fig. 3) after 5 min of exercise before training. The increases were significantly lower after training than before ($p < 0.05$ and 0.01, respectively). From the 5th min onwards the lactate concentrations gradually declined during the prolonged work but only after physical conditioning did muscle lactate approach the resting level.

Blood glucose averaged 4.0 $mmol \times l^{-1}$ at rest and no significant change occurred during exercise (Fig. 3). FFA concentration was 0.53 $mmol \times l^{-1}$ before exercise and after an initial lag it gradually increased and values around 1 $mmol \times l^{-1}$ were reached at the end of the exercise (Fig. 3). Physical conditioning did not result in any significant changes in blood glucose or FFA concentration either at rest or during exercise.

## Discussion

Heart rate, oxygen uptake and the R value changed during the prolonged work test as described earlier (Christensen 1931, Christensen and Hansen 1939). The change in these variables as well as in maximal oxygen uptake as a result of physical conditioning was in accordance with what could be expected from cross sectional and longitudinal training studies (Christensen 1931, 1932, Christensen and Hansen 1939, Bevegard, Holmgren and Jonsson 1963, Saltin *et al.* 1968).

The important new finding was that two months of physical training with a total of 6 h of running also resulted in a less pronounced depletion in muscle glycogen during exercise at the same absolute work load. This difference was not detectable in the early phase of the exercise but well established when comparisons are made for the whole work period.

Two major objections may be raised when making a comparison between the glycogen utilized before and after training. One is that the content of muscle glycogen at start of work was not the same although both values were within normal limits (Karlsson 1971). On the basis of the results in Bergström *et al.* (1967), where muscle glycogen was varied between 19 and 289 mmol glucose units $\times kg^{-1}$ wet muscle, it can be calculated that a difference of 20 mmol, as in the present study, does not significantly alter the glycolytic rate during work. This conclusion is further supported by the data for 3 of the subjects who had the same initial content of muscle glycogen before and after training. The 3 subjects demonstrated the same rate of glycogen utilization as all 5 subjects (Fig. 2).

The other problem is related to the fact that the final muscle biopsies were not taken at the same time of exercise (90 *vs* 120 min) in the 2 expts. Since a fairly constant glycolytic rate was observed after 20 min of work and since a complete glycogen depletion had not occurred in any subject the comparisons in Fig. 2 are valid. If the rate of glycogen utilization between 20 and 90 (observed or estimated) min is calculated this is found to be 0.41 $mmol \times kg^{-1} \times min^{-1}$ after as compared to 0.66 $mmol \times kg^{-1} \times min^{-1}$ before training ($p < 0.01$).

Any exact quantifications to what extent a reduced production of lactate and/or oxidation of pyruvate is present after physical conditioning cannot be settled on the basis of the present data. There is, however, indirect evidence of a less pronounced energy yield from both anaerobic and aerobic glycogen breakdown. 5 min after onset of the submaximal work muscle and blood lactate were lower as compared to before training. This could have been anticipated as it has been shown that muscle lactate is related to the oxygen deficit at start of exercise which in turn is a function of the relative work level (Karlsson 1971, Linnarsson *et al.* 1973). However, the lactate concentration depends not only on production but also on the turn-over in the body. It has been suggested that lactate is turned over faster in prolonged work after physical training (Karlsson and Jorfeldt 1971, Karlsson *et al.* 1972). In this study the relationship between the muscle and blood lactate concentrations was very similar in the two conditions implying an unchanged distribution and turn over rate for lactate. Consequently the lower muscle and blood lactate at onset of exercise after training might indicate that less glycogen has been used anaerobically.

The lower glycogen depletion and the lower R values during exercise in the presence of the same oxygen uptake point to a reduced oxidative use of carbohydrates after training. It has been proposed (Saltin and Karlsson 1971) that the enhanced fat oxidation and the reduced carbohydrate oxidation as indicated by the decrease in the R value observed in trained subjects are brought about by an increased recruitment of "red" (high oxidative—slow twitch—see Gollnick *et al.* 1972 for a further characterization) fibres. At the work levels used in this study (65 % of $\dot{V}_{O_2}$ max before and 59 % after training) it is highly probable that the slow twitch fibre are predominantly engaged regardless of the training status (Gollnick *et al.* 1973 a). Thus, the training effect does not seem to be the result of a change in recruitment pattern. Moreover, it has not been possible to demonstrate a change in the percentage of slow twitch fibres with physical training in humans (Gollnick *et al.* 1973 b). On the other hand, an enhanced oxidative capacity of all the fibres of human skeletal muscle has been observed after physical training (Gollnick *et al.* 1973 b). Furthermore, in animal muscle it has been demonstrated that along with the increase in oxidative enzymes goes an increase in the capacity to oxidize fatty acids (Mole, Oscai and Holloszy 1971). Thus, the decreased glycogen consumption observed in this study most probably is due to enhanced oxidative capacity which in turn leads to a higher capacity to oxidize fat.

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Acta physiol scand 1974 90 218—225

From the Departments of Experimental Ophthalmology and Histology University of Lund Sweden

# Uptake of Histamine or Histamine Metabolites into Sympathetic Nonadrenergic Axons

By

B Ehinger

Received 23 March 1973

## Abstract

Ehinger B *Uptake of histamine or histamine metabolites into sympathetic non adrenergic axons* Acta physiol scand 1974 90 218—225

Rabbit tissues were exposed to tritiated histamine or histidine either by intraocular injections (iris or by incubations (iris superior cervical ganglion) When the iris was exposed to tritiated histamine radioactivity appeared in a pattern very similar but not completely congruent to that of the adrenergic terminals The neuronal radioactivity disappeared after sympathetic denervation and also after decentralization of the superior cervical ganglion Application of tritiated histidine did not give rise to any neuronal radioactivity Sympathetic ganglion cells did not accumulate histamine under conditions when nerve fibres did The experiments suggest the possibility of a set of sympathetic nonadrenergic axons accumulating histamine or histamine metabolites If so these axons run close to the adrenergic terminals and presumably they arise in structures situated centrally of the superior cervical ganglion It is further concluded that there is under normal conditions no very efficient neuronal conversion of histidine to histamine in the uvea

Histamine has for a number of decades occupied a prominent position among substances with known and pronounced biological activities Nonetheless its physiological function is still poorly understood One hypothesis is that histamine acts as a chemical neurotransmitter Although never generally accepted there are arguments in favour of this view (see Green 1970 Tuttle and McCleary 1971 Ryan and Brody 1970 1972

It has been shown that exogenous histamine is accumulated in peripheral nerves (see Ryan and Brody 1970) Adrenergic neurons have a characteristic and highly efficient mechanism for recapturing their transmitter The location of the histamine uptake is therefore of prime interest for the interpretation of its significance but is nevertheless not known In view of the information obtained with autoradiographic studies on the uptake of putative and established neurotransmitters such as glycine GABA and catecholamines (see e g Ehinger 1972 Iversen and Bloom 1972 or Hökfelt and Ljungdahl 1972 for references) it was of interest to see if the uptake also of $^3$H histamine into peripheral tissues could be examined by autoradiography The results of such morphological studies are reported here

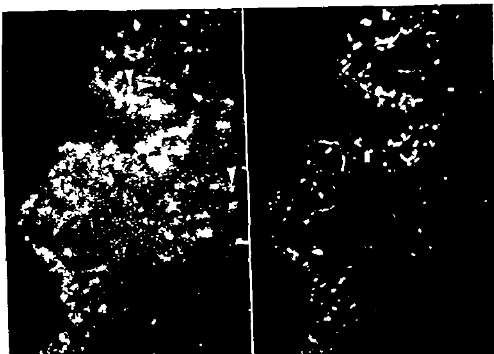


Fig 1 Autoradiogram and fluorescence picture of rabbit iris 4 h after the injection of 12 5 μCi $^3$H histamine intravitreally Left, dark field micrograph of the autoradiogram right, fluorescence micrograph showing the adrenergic nerves The silver grains in the autoradiogram appear as small white spots often coalescing to clusters The adrenergic fibres appear as bright strands or spots In most places there are dense accumulations of radioactivity in positions corresponding to the adrenergic nerves In a few places there are accumulation of radioactivity without any corresponding adrenergic fibre (e g at the arrows) × 185

## Material and methods

Albino rabbits weighing 1 5—2 kg were used in the study Tritiated histamine (1 0 Ci/mmole The Radiochemical Centre Amersham Buckinghamshire England) or histidine (52 2 Ci/mmole The Radicohemical Centre Amersham Buckinghamshire England) was administered either *in vivo* by intravitreal injection into the eye (12 5 μCi = 0 23 μg histamine in water or 50 μCi = 0 15 μg histidine in 2 % ethanol injection volume 50 μl) 4 h before the animals were killed or *in vitro* by incubating small iris tissue pieces (about 50 mg each) in 4 ml of a Krebs-Ringer bicarbonate solution at 37 C with $0\,36 \times 10^{-6}$ M $^3$H histamine The incubation solution was continuously mixed by mechanical shaking and was well aereated with 5 % $CO_2$ in oxygen After 30 min incubation the tissue pieces were rinsed in ice-cold Krebs Ringer bicarbonate solution 2 changes with 10 min in each They were subsequently quenched in a liquid propane propylene mixture cooled by liquid nitrogen, freeze dried fixed in gaseous formaldehyde by heating the tissue for 1 h at 80 C in a glass jar together with some paraformaldehyde (about 5 g paraformaldehyde in a 1000 ml jar) embedded directly in plastic (Durcopan ACM Fluka) by infiltrating them *in vacuo* in the monomer with appropriate additives for polymerization sectioned at 4 μm on an LKB Pyramitome and covered with autoradiographic stripping film (Kodak AR 10) The procedure has been shown to give good localization with negligible diffusion or extraction artefact in studies on the uptake of both amino acids Ehinger and Falck 1971 Ehinger 1972) and catecholamines (Ehinger and Falck 1971) The exposure times were 3 to 6 months

Sympathetic denervation of the iris was performed by excising the homolateral cervical sympathetic chain It has been shown that the procedure results in complete homolateral adrenergic denervation (Ehinger Falck and Rosengren 1969)




Fig 2



Fig 3

Fig 2 Autoradiogram of rabbit iris 4 h after 12 5 $\mu$Ci $^3$H histamine intravitreally Top dark field micrograph bottom phase contrast micrograph of the same area A nerve trunk (N) is strongly radioactive and also some spots (presumably nerves close to the vessel × 540

Fig 3 Autoradiogram of rabbit ciliary process 4 h after 12 5 $\mu$Ci $^3$H histamine intravitreally Top dark field micrograph bottom phase contrast micrograph of the same area The process is slightly swollen There is moderate radioactivity in the epithelium and a low radioactivity in the stroma × 540

## Results

After the intraocular injection of histamine a low to moderate radioactivity appeared diffusely in the ciliary body and in the iris stroma and a much stronger radioactivity in narrow strands and spots which were more densely located at the dilator muscle than elsewhere (Fig 1) Such strands and spots were also seen at large vessels and radioactivity was apparent in large nerve trunks (Fig 2) There was also usually a less marked though none the less distinct radioactivity in the ciliary epithelium particularly in the posterior processess (Fig 3) and also in the walls of large vessels There was no particular radioactivity in the iridic stromal cells in the sclera or in the cornea No cells classifiable as radioactive mast cells were observed Mast cells are known to be very rare in the rabbit iris (Levene 1962) In the chamber angle (i e the loose tissue around the spaces of Fontana) some highly radioactive spots and strands have been observed (Fig 4)

The distribution of the radioactivity so strongly suggested neuronal localization that a comparison was made with the distribution of the adrenergic terminals which



Fig 4 Autoradiogram of the chamber angle tissue rabbit Left, dark field micrograph right phase contrast micrograph of the same area. There are some strongly radioactive spots and strands (arrows) that may represent nerve fibres × 385

could be photographed before the application of the autoradiographic film The technical procedure with freeze-drying fixation in gaseous formaldehyde of appropriate humidity and subsequent anhydrous handling of the tissue gives strong and easily detectable fluorescence in the adrenergic neurons as is well known from the Falck and Hillarp histochemical method for catechol and indoleamines (Ehinger 1971) It was then observed (Fig 1) that the radioactivity was distributed in a pattern very similar to that of the adrenergic terminals However close analysis of a large number of picture pairs revealed that the two patterns were not accordant in all details Often an adrenergic fibre was observed with no concomitant radioactivity Less frequently there was also a radioactive spot or strand without any adrenergic terminal being observable (Fig 1)

The resolution of the radiographic method is not sufficient to judge whether the radioactivity is in the adrenergic terminal or a nearby structure such as the Schwann cell or a concomitant non adrenergic axon However after sympathetic denervation (3 animals) or after decentralization of the homolateral superior cervical ganglion (2 animals) all radioactive spots or strands suggestive of representing nerves disappeared In the decentralizations fluorescence microscopy according to Falck and Hillarp confirmed that the adrenergic axons remained intact Slides from the normal contralateral control eye showed normal radioactive nerve fibres




Fig. 2

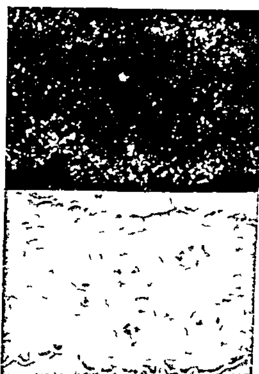


Fig. 3

Fig. 2. Autoradiogram of rabbit iris 4 h after 12.5 μCi $^{3}H$ histamine intravitreally. Top: dark field micrograph; bottom: phase contrast micrograph of the same area. A nerve trunk (N) is strongly radioactive and also some spots (presumably nerves) close to the vessel. × 540

Fig. 3. Autoradiogram of rabbit ciliary process 4 h after 12.5 μCi $^{3}H$ histamine intravitreally. Top: dark field micrograph; bottom: phase contrast micrograph of the same area. The process is slightly swollen. There is moderate radioactivity in the epithelium and a low radioactivity in the stroma. × 540

## Results

After the intraocular injection of histamine a low to moderate radioactivity appeared diffusely in the ciliary body and in the iris stroma, and a much stronger radioactivity in narrow strands and spots which were more densely located at the dilator muscle than elsewhere (Fig. 1). Such strands and spots were also seen at large vessels, and radioactivity was apparent in large nerve trunks (Fig. 2). There was also usually a less marked though none the less distinct radioactivity in the ciliary epithelium, particularly in the posterior processess (Fig. 3) and also in the walls of large vessels. There was no particular radioactivity in the iridic stromal cells, in the sclera or in the cornea. No cells classifiable as radioactive mast cells were observed. Mast cells are known to be very rare in the rabbit iris (Levene 1962). In the chamber angle (i.e. the loose tissue around the spaces of Fontana) some highly radioactive spots and strands have been observed (Fig. 4).

The distribution of the radioactivity so strongly suggested neuronal localization that a comparison was made with the distribution of the adrenergic terminal, which



Fig 7 Autoradiogram of rabbit ciliary processes 4 h after the intravitreal injection of 50 $\mu$Ci $^3$H histidine Phase contrast micrographs Left focus on the silver grains Right focus on the section Both layers of the epithelium are radioactive × 435

In the irides exposed to histidine radioactivity only appeared weakly and diffusely through the stroma and somewhat stronger in the ciliary epithelium particularly that of the posterior type (Fig 7) There was no sign of any neuronal localization of the radioactivity

## Discussion

The autoradiographic method used in the present study was specially developed for the study of the distribution of readily diffusible substances such as catecholamines and amino acids The tissues are freeze dried fixed in gaseous formaldehyde and embedded in plastic under anhydrous conditions The method was shown to give good localization with negligible diffusion or extraction artefacts (Ehinger and Falck 1971)

Uptake of histamine into postganglionic nerve trunks has been demonstrated previously (see Ryan and Brody 1970) but the precise localization of the uptake has not been established The present experiments unequivocally confirm that histamine or a histamine metabolite is accumulated in nervous structures when the tissue is exposed to histamine either *in vivo* or *in vitro* Subjectively judged from the higher radioactivity in similar experiments with tritiated noradrenaline (Ehinger unpublished) the uptake procedure seems less efficient for histamine than for noradrenaline The disappearance of the radioactivity upon sympathetic denervation and the striking similarity between the distribution of the radioactivity and of the adrenergic fibres strongly suggests that the uptake is into sympathetic axons In the iris at least mast cells are not involved This is of importance since the action of mast cells obscure the interpretations in many other tissues Mast cells are very scarce in the rabbit iris (Levene 1962)

Adrenergic terminals have a very efficient mechanism for accumulating catecholamines The present observations raise the question whether adrenergic terminals may also acumulate histamine No quantitative data appear to be available on the point A catecholamine releasing effect of histamine could indirectly suggest such an




Fig 5 Autoradiogram of rabbit iris incubated in $^{3}H$ histamine There is radioactivity in spots and strands at the dilator (top part) suggesting neuronal uptake (compare with Fig 1) There is also radioactivity in the muscle cells of a blood vessel (V) × 410

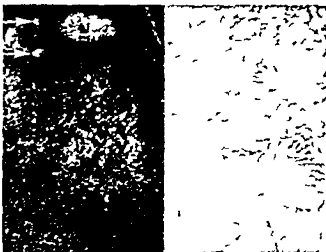


Fig 6 Autoradiogram of rabbit superior cervical ganglion incubated in $^{3}H$ histamine Left dark field micrograph right phase contrast micrograph There is strong radioactivity in a coarse nerve trunk (\) and two smaller nerve trunks (arrows) but else the radioactivity is moderate and diffusely distributed Ganglion cells occur in the bottom left hand corner The radioactivity is not different from that of the surrounding connective tissue × 235

The results of the iris incubation experiments were identical with the *in vivo* experiments (Fig 5) except that the total tissue radioactivity was less which called for the longest exposure times The uptake into the vessel walls (Fig 5) was normally more prominent in the incubation experiments than in the *in vivo* experiments

In sympathetic ganglia incubated in tritiated histamine radioactivity appeared in nerve trunks passing close to the ganglion but in the ganglion itself there was only a weak and diffusely distributed radioactivity with no difference between individual nerve cells or other tissue parts (Fig 6)

by histamine or by metabolites

Histamine is formed from histidine by decarboxylation The experiments with histidine were undertaken to see whether the radioactive precursor would cause an accumulation of radioactivity in the neurons (as is the case with the precursor L-dopa in adrenergic neurons) This was not the case and it seems that there is no very efficient conversion of histidine to histamine under normal conditions in the uvea

The experiments show that histamine or histamine metabolites can be accumulated in rabbit sympathetic peripheral iridic axons both *in vivo* and *in vitro* The axons are not necessarily adrenergic although they are sympathetic

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Acta physiol scand 1974 90 226—236

From the Department of Pharmacology Karolinska Institutet Stockholm Sweden

# Vascular and Metabolic Effects of Theophylline, Dibuturyl Cyclic AMP and Dibuturyl Cyclic GMP in Canine Subcutaneous Adipose Tissue in situ[1]

By

Bertil B Fredholm

Received 27 March 1973

## Abstract

Fredholm B B *Vascular and metabolic effects of theophylline dibuturyl cyclic AMP and dibuturyl cyclic GMP in canine subcutaneous adipose tissue in situ* Acta physiol scand 1974 *90* 226—236

In canine subcutaneous adipose tissue theophylline ($2 \times 10^{-4}$ M) cAMP and ATP (10 M) and DBcAMP ($8 \times 10^{-4}$ M) increased blood flow by by approximately 100 per cent. These compounds also antagonized sympathetic vasoconstriction. Theophylline and DBcAMP increased glycerol release dose-dependently while cAMP and ATP were ineffective up to 1 mM Theophylline (0 4—0.8 mM) potentiated the lipolytic effect of nerve stimulation while 2—8 mM apparently caused maximal stimulation *per se* DBcAMP did not affect FFA release following nerve stimulation while DBcGMP potentiated The apparent rate of re-esterification glucose uptake and lactate release was decreased by theophylline DBcAMP (0 1—0 4 mM) had no effect on these parameters while DBcGMP at the same concentration decreased re-esterification and lactate release Stimulated overflow of $^3$H from tissues prelabelled with L-$^3$H noradrenaline was reduced to 50 per cent by ATP (0 1—0 4 mM) but was unaffected by DBcAMP and DBcGMP at the same concentration The results support the view that cAMP mediates the metabolic actions of sympathetic nerve stimulation in canine subcutaneous adipose tissue The relationship between cAMP and vascular reactions may be more complex

*There is considerable evidence that cAMP mediates both increased lipolysis and* glycogenolysis following adrenergic $\beta$ receptor stimulation in white adipose tissue (*cf* Robison *et al* 1971) and it has been suggested that the vasodilatation following such stimulation is also mediated by cAMP (Robison *et al* 1971 Triner *et al* 1971 It has also been suggested that contraction of vascular smooth muscle following adrenergic $\alpha$ receptor stimulation is the consequence of a fall in intracellular cyclic AMP concentration (see Robison *et al* 1971) and the results obtained by

1 A preliminary account of some of these data was given at the meeting of the Scandinavian Pharmacological Society in Oslo (Acta pharmacol toxicol 28 suppl 1 p 47 1970)

Abbreviations used FFA = free fatty acids ATP = adenosine 5 triphosphate cAMP *or* cyclic AMP = adenosine 3 5 monophosphate DBcAMP or dibuturyl cyclic AMP = $N^6$ 2-O-dibuturyl adenosine 3 5 monophosphate DBcGMP *or* dibuturyl cyclic GMP = $N^6$ 2-O-dibuturyl guanosine 3 5 monophosphate EDTA = ethylene diamine tetraacetic acid

Triner *et al* (1971) showing decreased cyclic AMP formation in human gastro epiploic artery following such stimulation could be interpreted along these lines

In isolated perfused canine subcutaneous adipose tissue both metabolic and vascular events can be studied simultaneously Thus activation of the sympathetic nerve supplying the canine subcutaneous adipose tissue leads to vasoconstriction increased lipolysis and glycogen breakdown (*cf* Fredholm 1970) The vasoconstriction is changed to a vasodilatation after treatment with an adrenergic $\alpha$ receptor blocking agent The vasodilatation as well as the lipolysis and glycogenolysis is abolished by adrenergic $\beta$ receptor blocking agents (Ngai *et al* 1966 Fredholm and Rosell 1968 Fredholm and Karlsson 1970)

In the present experiments the vascular and metabolic effects of cAMP dibutyryl cAMP (Posternak *et al* 1962) and theophylline a wellknown inhibitor of cyclic nucleotide pho phodiesterase (Butcher and Sutherland 1962) were studied in the isolated perfused dog subcutaneous adipose tissue *in situ* The doses of these agents required to produce vasodilatation and lipolysis were compared as were the changes in the responses with time in order to study the question if both responses are likely to have the same cause The responses of the adipose tis ue to cAMP dibutyryl cAMP theophylline as well as to dibutyryl cyclic GMP and ATP were compared to the known effects of sympathetic nerve stimulation before and after adrenergic receptor blockade (*cf* Fredholm 1970) The interactions between these drugs and nerve stimulation was also investigated

## Materials and methods

The experiments were performed on 51 fed female mongrel dogs weighing 6—19 kg Anesthesia was induced with sodium pentobarbital (Nembutal® Abbott) 25—30 mg/kg i v and was supplemented with single i v injections of 25—30 mg Heparin (5000 IU/ml Vitrum) was administered i v (appr 0 5 ml/kg B W ) to prevent clotting Subcutaneous adipose tissue was isolated from surrounding tissues as described elsewhere (Rosell 1966)

In four dogs the tissue was perfused at a constant rate with the dog s own blood with a previously described perfusion apparatus (Renkin and Rosell 1962) The venous blood was directed via a drop-counter into ice cooled centrifuge tubes

In seven dogs the tissue was autoperfused by directing blood via a drop-counter from the femoral artery to the tissue which was enclosed in a plethysmograph filled with body warm Tyrode solution The volume of the enclosed tissue was continuously monitored by a piston recorder writing on a smoked drum. The vein was cannulated by a w de bore plastic catheter the free end of which could be placed at any desired le el abo e the preparation By a sudden elevation of 10—15 cm the tissue volume rose at first rapidly later at a constant rate This later phase can be used to estimate the hydrodynamic conducti ity of the tissue (Cobbold *et al* 1963) The capillary filtration coefficient (CFC) which is expre sed n the units ml fluid f ltered per min per 100 g tissue per mm Hg pressure differenc can be calculated by assuming that 4/5 of the venous pressure elevation is transmitter to the capillar es The application of the method for stud es of va cular reactions in canine subcutaneous adipose tissue has previousl been described (Öberg and Rosell 1967 Fredholm *et al* 1970) In the remain ng 41 dogs th tissue was autoperfused and venous blood was returned directly to the animal via the femoral vein

The blood pressu e was measured by a Statham Model PC 23 transducer connected to the drop-counter and recorded together with the blood flow on a Grass Model 5B-polygraph The nerve to the tissue was cut between l gatures at the level of the external hiatus of the ngu nal canal The nerve was placed on a bipolar s l er electrode protected from drying by plastibase (Squibb) and st mulated with pulse of supramaximal intensity (11—13 V ) and duration (2 ms) del ered at the rate of 4 Hz from a Grass Model 4B stimulator

Acta physiol scand 1974 90 226—236

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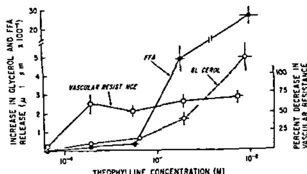
In canine subcutaneous adipose tissue theophylline ($2 \times 10^{4}$ M) cAMP and ATP ($10^{5}$ M) and DBcAMP ($8 \times 10^{4}$ M) increased blood flow by by approximately 100 per cent. These compounds also antagonized sympathetic vasoconstriction Theophylline and DBcAMP increased glycerol release dose-dependently while cAMP and ATP were ineffective up to 1 mM Theophylline (0 4—0 8 mM) potentiated the lipolytic effect of nerve stimulation while 2—8 mM apparently caused maximal stimulation *per se* DBcAMP did not affect FFA release following nerve stimulation while DBcGMP potentiated The apparent rate of re-esterification glucose uptake and lactate release was decreased by theophylline DBcAMP (0 1—0 4 mM) had no effect on these parameters while DBcGMP at the same concentration decreased re-esterification and lactate release Stimulated overflow of $^{3}$H from tissues prelabelled with L $^{3}$H noradrenaline was reduced to 50 per cent by ATP (0 1—0 4 mM) but was unaffected by DBcAMP and DBcGMP at the same concentration The results support the view that cAMP mediates the metabolic actions of sympathetic nerve stimulation in canine subcutaneous adipose tissue The relationship between cAMP and vascular reactions may be more complex


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Fig 1 The effect of theophylline on vascular resistance (□——□) in adipose tissue (per cent change relative to preinfusion control) and on glycerol (O O) and FFA (●——●) release rate (increase in μmol × min⁻¹ × 100 g⁻¹ over preinfusion control values) The values represent the mean ± SE of the changes induced following 10—15 min infusion of theophylline Each point is the mean of 3—10 trials




## Results

### *Theophylline*

Theophylline ($2 \times 10^{-4}$ M—10 M) increased the blood flow to 20—60 ml × min⁻¹ × 100⁻¹ g from a resting value of 11 4 ± 2 9 ml × min⁻¹ × 100 g⁻¹ This corresponds to the maximal blood flow rates obtainable with other drugs such as isoprenaline acetylcholine prostaglandin E histamine and bradykinin (Fredholm *et al* 1970)

Theophylline also induced an approximately 100 per cent increase in the capillary filtration coefficient (CFC) (Table I) This increase was larger than that produced by nerve stimulation Na EDTA which like methylxanthines alters the metabolism of intracellular calcium gave similar increases in CFC Nerve stimulation superimposed upon a theophylline infusion appeared to further increase in CFC but this effect was not statistically significant The vasoconstriction induced by nerve stimulation was antagonized by theophylline and Na EDTA in a dose dependent manner (Table II)

Theophylline produced a dose dependent increase in glycerol and FFA release in the denervated adipose tissue (Fig 1) The rate of FFA release during infusion of high concentrations ($2 \times 10^{-3}$ M or more) of theophylline was 2—6 times higher than the rate of glycerol released on a molar basis The changes with time are illustrated in Fig 2 which summarizes the findings from five experiments in which high concentrations of theophylline ($5—12 \times 10^{-3}$ M) was present in the blood perfusing the adipose tissue After about 15 min the rate of glycerol release reached a constant level which was maintained for the entire period The FFA release continued to rise for 20 min following the start of the theophylline infusion The rate of FFA release was less than 3 times the rate of glycerol release for the first 5 min while it was higher from 10 min on

Blood flow rose markedly in the first minutes after the start of the infusion of theophylline and reached its peak values before 5 min whereafter it returned slowly towards control values (Fig 2) After cessation of the infusion there was a further rapid fall in blood flow (not shown in Fig 2) Concomitantly with the rise in the blood flow there was an increase in glucose uptake and lactate release but af

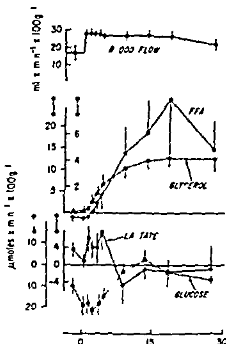



Fig 2

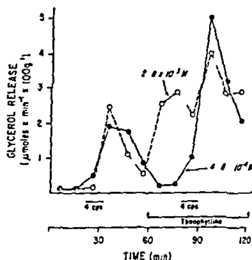


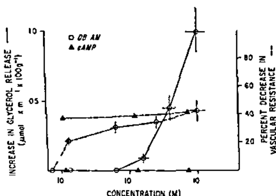

Fig 3

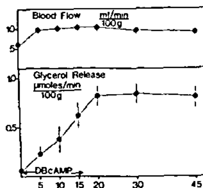
Fig 2 The effect of theophylline (5—12 mM in blood) on blood flow FFA release glycer release lactate release and on glucose uptake in canine subcutaneous adipose tissue Mea ±SE from 5 expts on the abscissa time in minutes Theophylline infusion was started a time 0 In the panel showing the FFA release is plotted both the actually observed FFA relea (●——●) and the calculated rate of formation (3 × glycerol release rate (● ●) A roug indication of the rate of re-esterification can be obtained by subtracting the former from th latter

Fig 3 The effect of nerve stimulation (4 Hz 12 V 2 ms for 10 min) on the rate of glycero release before and during infusion of theophylline ●——● 0 4—0 8 mM theophylline in bloo during the second 60 min period O— —O 2—8 mM theophylline in blood during the secon 60 min period Mean of 5 expts in each group

10 min the glucose uptake had fallen ($p < 0\,01$) to practically zero and lactate was extracted from the blood stream instead of being released ($p < 0\,01$)

Lipolysis following sympathetic nerve stimulation (4 Hz for 10 min) was studied before and during theophylline infusion in ten animals The results are summarized in Fig 3 0 4—0 8 $10^{-3}$ M theophylline approximately doubled the maximal lipolytic rate following sympathetic nerve stimulation A 10 times higher dose of theophylline was strongly lipolytic *per se* After 20 min infusion when a steady level of release was obtained (Fig 2) a nerve stimulation was superimposed This produced a decrease in the release rate but after cessation of the stimulation there was an increase Nerve stimulation did not significantly alter the overall release of glycerol since the increase after stimulation was not significantly different from the decrease during stimulation The fact that sympathetic nerve stimulation did not cause an




F g 4

Fig 5

Fig 4 Effect of increasing concentrations of dibuturyl cyclic AMP and cyclic AMP on ad pose tissue vascular resistance (per cent change from pre infusion control) and on glycerol release rate (increase in $\mu$mol$\times$min$^{-1}\times$100 g$^{-1}$ over preinfusion values) The values for DBcAMP repre ent mean$\pm$S E of the changes induced by 10—15 min infusion of the drugs (3—10 dogs) and for cAMP means of the changes in 2—4 dogs

F g 5 The effect of dibuturyl cyclic AMP (1 5—5 $\mu$mol$\times$ml blood$^{-1}$) on blood flow (ml$\times$min$^{-1}\times$100 g$^{-1}$) and on glycerol release rate ($\mu$mol$\times$min$^{-1}\times$100 g$^{-1}$) during and after an infusion lasting for 15 min Mean$\pm$S E of 3 expts The figures along the abscissa represent time in minutes from the start of the infusion

additional increase in glycerol release probably indicates that theophylline had caused a maximal stimulation of lipolysis (*cf* Weiss *et al* 1966)

*Nucleotides*

In three dogs DBcAMP cAMP and ATP were administered in graded doses as clo e i a injections A fifty per cent increase in blood flow was produced by approximately $3\times10^{-6}$ mol DBcAMP $2\times10^{-7}$ mol cAMP and by 2 $10^{-9}$ mol ATP Thus cAMP appeared to be a more potent vasodilator than its dibuturyl derivative During constant rate infusion of the nucleotides a similar relationship between the two drugs was observed (Fig 4) The vasodilatation produced by DBcAMP 1—5$\times10^{-3}$ M developed over a period of several minutes and reached its maximum towards the end of a 15 min infusion (Fig 5) Following the cessation of the infusion the blood flow returned only slowly to preinfusion level which were not reached even half an hour later

Although cAMP produced significant vasodilatation already at $2\times10$ M there was no significant effect on lipolysis even at a 50 times higher concentration (Fig 4) Vasodilatation was produced by $2\times10^{-5}$ DBcAMP but a 10 times higher concentration was needed to ee a significant increa e in lipolysis (Fig 4)

The glycerol relea e increased slowly during the infusion of DBcAMP and the highest level was obtained after the end of a 15 min infusion On the other hand the lipolytic rate remained elevated for a considerable period of time (Fig 5) DBcAMP had no effect on lactate relea e from adipo e tissue but caused a sligh increase in the

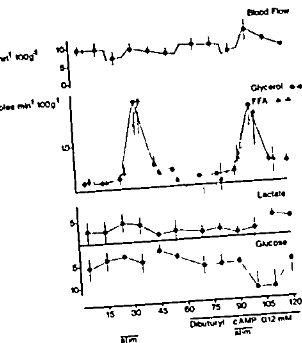



Fig 6 The effect of dibuturyl cyclic AMP (0 12 mM) and s m pathetic nerve stimulation (4 Hz 12 V 2 ms for 10 min) on blood flow glycerol (O——O) and FFA (△ △) release lactate release and glucose uptake Mean±S E from 4 expts Time in minutes from the start of the experiment along the abscissa

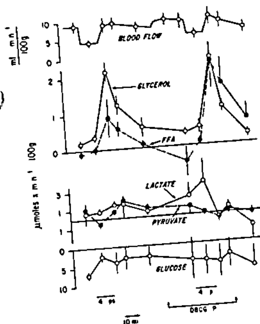



Fig 7

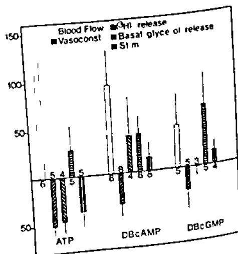



Fig 8

glucose uptake (Fig 6) The latter effect was essentially parallel to the blood flow increase which might have been the cause DBcAMP had no significant effect on the glycerol or FFA release induced by sympathetic nerve stimulation (Fig 6)

DBcGMP had no effect on the glucose uptake but increased the evoked FFA release (Fig 7) The effect on FFA release indicate a decreased re-esterification As was the case with theophylline evidence of decreased re esterification coincided in time with decreased lactate release

Fig 8 summarizes the effects of ATP DBcAMP and DBcGMP All compound were significantly vasodilator *per se* and they all inhibited the vasoconstrictor response to nerve stimulation The cyclic nucleotide derivatives both significantly increased basal lipolysis but had no effect on the evoked glycerol release or on the release of $^3H$ radioactivity reflecting transmitter overflow from adipose tissue It is of interest to note that ATP inhibited the release of $^3H$ radioactivity and the evoked responses *i e* vasoconstriction and glycerol release to an approximately equal degree

## Discussion

In the present experiments DBcAMP and theophylline produced lipolysis in concentrations similar to those needed to produce the same response in rat adipose tissue or adipocytes *in vitro* (Bieck *et al* 1969 Blecher 1971 Weiss *et al* 1966) cAMP was ineffective even at 1 mM concentration in agreement with *in vitro* data (Bieck *et al* 1969) DBcGMP was a better lipolytic agent in the present experiments than could be predicted from *in vitro* data on the relative potency of cAMP and cGMP (Blecher *et al* 1971 Murad *et al* 1969) It has been reported that cGMP has a mixed action it stimulates protein kinase directly and it inhibits the enzymatic degradation of cAMP (Murad *et al* 1969) An indirect mechanism of action of DBcGMP via inhibition of phosphodiesterase was indicated in the present experiments by the finding that the drug potentiated FFA release following nerve stimulation like theophylline DBcAMP in the doses used had no effect on the FFA release following

Fig 7 The effect of dibutyryl cyclic GMP (0 1—0 4 mM) on blood flow glycerol (O——O) and FFA (△ △) release lactate release pyruvate release and glucose uptake Mean±S E from 4 expts

Fig 8 Some effects of ATP (0 1—0 5 mM) dibutyryl cyclic AMP (0 1—0 5 mM) and dibutyryl cyclic GMP (0 1—0 4 mM) expressed as the mean±S E of the per cent changes in individual experiments Figures at the bottom of the bars represent number of determinations The blood flow response is the per centage change in blood flow after 5 min infusion relative to the flow immediately before the start of the infusion Blood flow in the different control groups was ATP—7 2±1 6 DBcAMP—11 8±0 8 DBcGMP—10 9±1 5 ml×min $^{-1}$×100 g $^{-1}$ The vasoconstrictor response is the relative change in vascular resistance induced by sympathetic nerve stimulation (4 Hz 12 V 2 ms) The ($^3H$ release refers to the total net overflow of ($^3H$) radioactivity induced by an identical nerve stimulation (for details Fredholm and Hedqvist 1972) The basal glycerol release is the release of glycerol occurring 10—14 min after the start of the infusion relative to the resting glycerol release during the control period (ATP—0 20±0 06 DBcAMP—0 25±0 06 DBcGMP—0 18±0 07 mol min $^{-1}$×100 g $^{-1}$) Stimulated glycerol release refers to the total net release of glycerol induced by sympathetic nerve stimulation (Control values ATP 1 15±0 46 DBcAMP—1 80±0 32 DBcAMP—2 07±0 23 mol×100 g $^{-1}$×min of stimulation $^{-1}$)

nerve stimulation suggesting that its actions were mainly direct Blecher and Hunt (1972) recently found that deacylation of dibutyryl cyclic AMP is the ratelimiting step in the metabolism of dibutyryl cyclic AMP in several rat tissues The time course of vasodilatation and lipolysis following infusion of DBcAMP in the present experiments is compatible with the hypothesis that DBcAMP is not active by itself but has to be deacylated to active metabolites the formation of which is rate limiting for the development of the responses

Following maximal doses of theophylline and—to a lesser degree—following DBcGMP re esterification decreased as evidenced by an increase in the ratio between the rate of FFA and glycerol release It is of interest that in these situations there was also a fall in the release of lactate There is a parallelity between the intracellular lactate concentration and the degree of re esterification in canine subcutaneous adipose tissue in several conditions (Fredholm 1971 1972 Fredholm Linde and Persson 1973) including sympathetic nerve stimulation before and after adrenergic *α* receptor blockade (Fredholm and Karlsson 1970) This connection between lactate levels and re esterification may depend on the red ox state of cytoplasmatic NAD (Fredholm 1970 1971) It is therefore noteworthy that theophylline causes a marked rise in oxygen uptake in adipose tissue (Fredholm *et al* 1973) as does sympathetic nerve stimulation after adrenergic *α* receptor blockade

While the metabolic actions of theophylline are similar to those of adrenergic *β* receptor activation (*cf* Fredholm and Karlsson 1970) there are qualitative differences in the vascular effects Thus stimulation of adrenergic *β* receptors cause a small (0—40 %) change in CFC while theophylline caused an increase of about 100 per cent although flow increases were similar The effect of isoprenaline was similar to that obtained by prostaglandin $E_1$ acetylcholine and poststimulatory hyperemia while the effects of theophylline was more like the effect of histamine bradykinin or EDTA which are known to increase vascular permeability This indicates that at least in the case of the vasculature in adipose tissue the actions of theophylline and adrenergic *β* receptor stimulation are not all effected by a common mediator such as cAMP

There are also quantitative differences Thus vasodilatation was produced by theophylline and DBcAMP in much lower concentration than those needed for demonstrable metabolic activity and cAMP was at least as potent as DBcAMP as a vasodilator although it had no metabolic effects over the dose range studied It is possible that these differences could be due to differences in the degree of inhibition of phosphodiesterase by theophylline or in the rate of penetration for example of the cyclic nucleotides between different tissues Available data indicate that the phosphodiesterase inhibiting effect of theophylline in fat and vascular smooth muscle is quite similar however (*e g* Sheppard Wiggan and Tsien 1972 Weiss *et al* 1966 Triner *et al* 1971) It is also a well known fact that adenosine and its derivatives are highly potent vasodilators *per se* Therefore the mechanism by which these drugs cause vascular and metabolic effects in canine subcutaneous adipose tissue might be different

In the present experiments dibutyryl cyclic AMP dibutyryl cyclic GMP and theophylline all inhibited vasoconstriction induced by sympathetic nerve stimulation Results from a previous series of experiments indicate that isoprenaline might also inhibit the vasoconstrictor response (Fredholm *et al* 1970) It is quite possible that the inhibition of vasoconstrictor response by these drugs was at least partly due to stimulation of metabolism and the consequent accumulation of as yet unidentified vasodilator metabolites

The present finding that ATP also inhibited the vasoconstrictor response might indicate that the effect of dibutyryl cyclic AMP dibutyryl cyclic GMP and theophylline is nonspecific The inhibition of vasoconstriction by ATP was accompanied by an inhibition of $^3$H noradrenaline release which was not seen during infusion of dibutyryl cyclic AMP or GMP however Indeed inhibition of transmitter release might have been the cause of the inhibition of sympathetically induced vasoconstriction and glycerol release by ATP since all three processes were affected to approximately the same degree This inhibition of transmitter overflow is of considerable interest since the transmitter is stored together with ATP in nerve and medullary granules (Stjarne 1964) and the two might also be released together

In conclusion the present results are compatible with the theory that the metabolic effects of adrenergic $\beta$ receptor stimulation in canine subcutaneous adipose tissue *in situ* are mediated by increased cyclic AMP levels in fat cells It remains to be shown however whether this is the only mechanism of catecholamine activation of metabolism in adipose tissue *in vivo* It is clear from earlier experiments that simultaneously occurring vascular events strongly modify the metabolic response to sympathetic nerve stimulation (*cf* Fredholm 1970) The question whether these vascular events vasoconstriction before and vasodilatation after adrenergic $\alpha$ receptor blockade are mediated by changes in cyclic AMP levels in vascular smooth muscle cells in adipose tissue could not be directly answered by the present experiments The results do however suggest that cyclic AMP might inhibit the vasoconstrictor response to sympathetic nerve stimulation If this is a direct interaction at the level of the contractile apparatus or secondary to stimulation of metabolism in fat cell and stromavascular cells as well as the physiological importance of such inhibition in the regulation of adipose tissue blood flow remains to be elucidated

The present study was supported by grants from the Swedish Medical Research Council 40X 2553 and 40P 3828 The skillful technical assistance of Mr I an Sand ll Miss Margareta Stensdotter and Miss Gun lla Wikberg is gratefully acknowledged

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BLECHER M Biological effects and catabolic metabolism of 3 5-cyclic nucleotides and derivatives in rat adipose tissue and liver *Metabolism* 1971 *20* 63—77

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There are also quantitative differences. Thus vasodilatation was produced by theophylline and DBcAMP in much lower concentration than those needed for demonstrable metabolic activity and cAMP was at least as potent as DBcAMP as a vasodilator although it had no metabolic effects over the dose range studied. It is possible that these differences could be due to differences in the degree of inhibition of phosphodiesterase by theophylline or in the rate of penetration, for example, of the cyclic nucleotides between different tissues. Available data indicate that the phosphodiesterase inhibiting effect of theophylline in fat and vascular smooth muscle is quite similar however (e.g. Sheppard, Wiggan and Tsien 1972, Weiss *et al.* 1966, Triner *et al.* 1971). It is also a well known fact that adenosine and its derivatives are highly potent vasodilators *per se*. Therefore the mechanism by which these drugs cause vascular and metabolic effects in canine subcutaneous adipose tissue might be different.

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# Pulmonary Vascular Effects of Suddenly Induced Unilateral Blood Platelet Aggregation

B

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## Abstract

Bo G and J Hognestad *Pulmonary vascular effects of suddenly induced unilateral blood platelet aggregation* Acta physiol scand. 1974 90 237—243

Embolization of one or both lungs was achieved in cats by intravascular infusions of a collagen suspension. On intravenous collagen infusion, with platelet aggregates reaching the vasculature of both lungs large pulmonary pressor responses were obtained. Unilateral platelet aggregation in blood to the lungs was achieved by infusion of collagen into the right pulmonary artery. The resulting pressure/flow response indicated that blood flow was diverted to the contralateral lung. There was no indication that the pulmonary vascular resistance was increased in this contralateral non-embolized lung. It appears therefore that only vasculature in the lung which received platelet emboli is involved in the vasoconstriction following unilateral embolization of this type

Miliary pulmonary embolization releases several reactions in the lung and the mechanism behind some of these reactions are still debated. Thus there is no agreement as to how and to what extent pulmonary vasoconstriction may be elicited by pulmonary microembolization. Experimental observations and interpretations in this field have varied considerably. This may be explained to some extent by the fact that a great variety of substances has been used as embolizing material.

After miliary embolization with barium sulphate in dogs Price *et al* ([illegible]) found that a widespread generalized pulmonary vasoconstriction took place. They concluded that this vasoconstriction was largely the result of reflexes released from small pulmonary vessels. Others such as Daly *et al* (1931) who used lycopodium spores have not observed any increase in pulmonary vascular resistance upon microembolizing the lungs of dogs.

In a recent study (Bo and Hognestad 1972) we have shown that intravascular platelet aggregation achieved by intravenous infusion of a collagen extract created a very marked increase in the pulmonary vascular resistance (PVR). This increase in resistance was reversible and it appeared probable that both mechanical plugging of vessels and a considerable vasoconstriction contributed to the response observed.

The same experimental approach was used in the work reported here as a means of creating sudden microembolization of the pulmonary vasculature Unilateral infusions of collagen were undertaken with the intention of clarifying if generalized pulmonary vasoconstriction also involving the non-embolized lung was provoked by such infusions

## Materials and methods

A total of 7 cats weighing 2 5 to 4 kg were used They were anesthetized by intraperitoneal injections (30 mg/kg) of sodium pentobarbitone (Nembutal® Abbott)

*Ventilation* After tracheotomy a muscle relaxing substance Alloferin (1/2 mg/kg) was given and positive pressure ventilation started with a piston pump respirator (The Ideal *Respiration Pump C F Palmer Ltd London*) The pump frequency was 21 strokes per min. With the use of water seals the inspiratory peak pressure and the end tidal pressure were kept at 6 to 8 and 2 cm of water respectively The tidal volume of the respirator was adjusted so as to keep the arterial pH close to 7 40 Standardized careful hyperinflations of the lungs were carried out at regular intervals and between all tests with collagen infusions

*Experimental procedures* The thorax was opened by a sternum splitting incision Polyethylene catheters were introduced into the femoral artery and the left atrium for recording of the femoral arterial pressure ($P_{FA}$) and the left atrial pressure ($P_{LA}$) respectively A catheter was led through the wall of the right ventricle into the main pulmonary artery for measurement of pulmonary arterial pressure ($P_{PA}$) Statham P23Gb P23Db and P23De pressure transducers were used for the three pressure recordings mentioned above A flow probe was placed around the ascending aorta and aortic flow was recorded with a Nycotron square wave flow meter (type 372 Nycotron A/S Norway) The pressure transducers and the flow meter were connected to a six-channel Sanborn recorder (Model 350 Sanborn Co California) The body temperature of the animals was maintained at a normal level by placing them on a heated table and covering them with a polyethylene sheet

A thread was placed around the left pulmonary artery after careful blunt dissection Before any infusion was begun the left pulmonary artery was occluded with the use of this thread In this way it became possible to evaluate the pulmonary vascular resistance in the right lung in two situations with normal flow and with the flow through the lung approximately doubled

Total pulmonary vascular resistance (PVR) was calculated according to the following formula

$$PVR = \frac{P_{PA} - P_{LA}\ (\text{mm Hg})}{\text{Flow}\ (= \text{aortic flow})\ (\text{ml/min})}$$

*Infusions of collagen* 1/2 ml of a collagen suspension (prepared as described by Holmsen (1969)) was infused in the course of one min In 4 animals the following procedures were carried out in connection with the infusions

1 The left pulmonary artery was occluded and $P_{PA}$ $P_{LA}$ and aortic flow recorded during this situation Thereby the vascular resistance of the right lung could be obtained at a time when the flow through this lung was doubled
2 Infusion of collagen into the artery of the right lung was then carried out whilst the left pulmonary artery was kept closed The left pulmonary artery was opened up again when the infusion had been finished and the pressure and flow values were closely followed
3 The whole infusion procedure was repeated when the pressure and flow values had returned to normal levels
4 Finally an infusion of collagen into the femoral artery was carried out

In 2 animals alternate infusions of collagen into the left pulmonary artery and into the inferior caval vein were carried out In another animal intravenous collagen infusions were done at 3 varying distances from the lung i e into the caval vein into the right ventricle and into the main pulmonary artery These tests were performed in order to examine if the extent of the collagen effect was dependent on the intravascular site of infusion

Histology was performed as described in a previous work (Bo and Hognestad 1972)

## Results

The reactions in 4 animals (A B C D) to closing off one pulmonary artery to unilateral pulmonary infusions of collagen and to intra arterial infusion of collagen are shown in Table I

TABLE I Effects of 3 procedures on $P_{PA}$ and PVR in 4 animals
1 Clamping of left pulmonary artery
2 Infusion of collagen into right pulmonary artery
3 Infusion of collagen into aorta
(Q and $P_{LA}$ did not change during these experimental procedures.) For comparison the rise in PVR after clamping of the left pulmonary artery is calculated as if blood still went through both lungs.

| | | $P_{PA}$ rise in mmHg | PVR in % of the control value |
|---|---|---|---|
| A | Control situation | 12 | 100 |
| | After clamping of left pulm. artery | 12–15 | 150 |
| | After infusion into right lung | 12–15 | 150 |
| | After 2nd | 12–15 | 150 |
| | After intra arterial infusion | 12–20 | 233 |
| B | Control situation | 12 | 100 |
| | After clamping of left pulm. artery | 12–14 | 139 |
| | After infusion into right lung | 12–14 | 139 |
| | After 2nd | 12–14 | 139 |
| | After intra arterial infusion | 12–12 | 100 |
| C | Control situation | 15 | 100 |
| | After clamping of left pulm. artery | 15–20 | 150 |
| | After infusion into right lung | 15–18 | 130 |
| | After 2nd | 15–18 | 130 |
| | After intra arterial infusion | 15–22 | 170 |
| D | Control situation | 24 | 100 |
| | After clamping of left pulm. artery | 24–35 | 158 |
| | After infusion into right lung | 24–30 | 132 |
| | After 2nd | 24–30 | 132 |
| | After intra arterial infusion | 24–33 | 147 |

Collagen infusion into the artery of one lung appeared to cause a marked local vasoconstriction approaching an occlusion of the vascular supply to that lung. This could be deduced from comparisons between the effect of collagen infusion into one lung and the effect of a mechanical occlusion of the vascular supply to one lung. Closing off the left pulmonary artery resulted in an average rise in $P_{PA}$ of 4 mm Hg (30 %). Since cardiac output and $P_{LA}$ remained unchanged, there was a mean lowering by 25 % of the pulmonary vascular resistance in the right lung proper. When PVR was calculated as if both lungs had been perfused, a rise in this parameter to about 150 % was obtained (Table I).

Infusion of collagen into the right lung resulted in an average rise in $P_{PA}$ by 22 %. Since cardiac output and $P_{LA}$ were unchanged, there was consequently a mean rise in PVR to 137 % of the initial value. In no experiment did the $P_{PA}$ after unilateral collagen infusion rise above the value obtained by closing off the other pulmonary artery. Repeated unilateral collagen infusions gave reproducible responses.

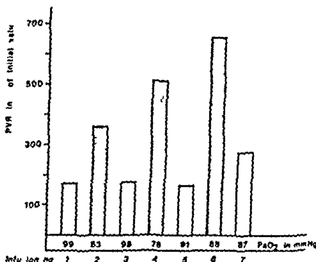



Fig 1 The increase in pulmonary vascular resistance and the changes in $PaO_2$ in one animal in response to alternate infusions of collagen into the pulmonary artery of the left lung ▢ and into the caval vein ■

Finally one infusion of collagen was given i a In 3 animals this caused a mean rise in PVR to 183 % of the initial value In one animal no response was obtained after such i a collagen infusion

In 2 animals successive infusions of collagen were given alternately into the left pulmonary artery and into the inferior caval vein Infusions at the latter site gave the more marked PVR responses which also showed a stepwise increase from one test to the next one Results from the tests in one of these animals are shown in Fig 1 *The PaO fell subsequent to the infusions and most markedly after the infusions* into the caval vein

Table II gives the responses to collagen infusions at various sites in one animal The pressure responses were small when collagen was infused into the main pulmonary artery When collagen was infused into the right ventricle however the responses were of the same order of magnitude as those obtained after intravenous infusions

*Morphology* The lungs from the 4 animals which had received two one sided collagen infusions and one intra arterial infusion of collagen were examined histologically In the right lungs which had received the two direct doses of collagen platelet aggregates were found in small arteries Collagen fibrils were frequently seen in the central parts of such aggregates In the left lungs which had received no direct collagen dose platelet aggregates were occasionally seen in the vessels However, collagen fibrils were never observed

## Discussion

In a previous publication (Bo and Hognestad 1972) we have shown that a marked rise in PVR occurs in the lungs of animals infused with a collagen extract Iv injections of a collagen suspension simulates microembolization by creating small platelet aggregates which lodge in small arterial vessels and in capillaries in the lung

TABLE II Increase in pulmonary arterial pressure ($P_{PA}$ mm Hg) and percentage rise in the pulmonary vascular resistance (PVR) subsequent to infusions of collagen in one animal at various distance from the pulmonary vascular bed The numbers indicate the order of injections

| Site of infusion | $P_{PA}$ rise in mmHg | PVR achieved as of initial value |
|---|---|---|
| 1 Caval vein | 14–20 | 420 |
| 2a Right ventricle | 16–34 | 306 |
| b repeated | 17–33 | 300 |
| 3a Into the pulmonary artery between the bifurcation and the pulmonary valves | 17–20 | 134 |
| b Into the pulmonary artery between the bifurcation and the pulmonary valves repeated | 19–25 | 152 |
| c Into the pulmonary artery between the bifurcation and the pulmonary valves repeated | 16–21 | 154 |
| 4 Caval vein | 14–24 | 211 |
| 5 Right ventricle | 14–23 | 200 |

The pulmonary vascular responses to experimental lung embolism appear to depend on at least two important factors namely the size and the quality of the material injected The physiological abnormalities in patients dying from pulmonary emboli are those of general circulatory shock combined with pulmonary hypertension and right heart failure Although mechanical obstruction to flow undoubtedly contributes to the emboli induced pulmonary hypertension several investigators have suggested that vasoconstriction may contribute more markedly to this pressor response The pressor response is apparently dependent on whether the emboli lodge in large arteries or in small ones There seems to be general agreement that large emboli trapped in the larger arteries of the lung do not create much concomitant vasoconstriction Autopsy studies (Dexter 1965) have shown as much as 65 to 70 per cent reduction in pulmonary arterial volume in patients who died from lung emboli In animals occlusion of a dominant fraction of the larger lung arteries is necessary in order to produce pulmonary hypertension (Hyland *et al* 1963 a)

When it comes to smaller emboli lodged in the minor arterial vessels there is however no general agreement as to the mechanisms involved It has been claimed that localized injections of certain small sized particulate materials (particles less than 100 $\mu$m) into the lungs of animals may cause generalized pulmonary hypertension and death (Price *et al* 1955) Evidence has been presented for such responses being due to a neurally mediated reflex constriction of smooth muscles in the lung vessels (Price *et al* 1955 Niden and Aviado 1956 Dunn 1920 Hyland *et al* 1963 b) Other workers doubt that such reflexes participate in the response pattern (William

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# The Role of Blood Platelets in Pulmonary Responses to Microembolization with Barium Sulphate

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**Abstract**

Bo G J Hognestad and J Vaage *The role of blood platelets in pulmonary responses to microembolization with barium sulphate* Acta physiol scand 1974 *90* 244—251

*Intravenous administration to cats of a barium sulphate suspension caused a temporary and* marked increase in the pulmonary vascular resistance On repeated infusions there was a gradual decrease in this pulmonary response At the same time there occurred a marked fall in the number of circulating blood platelets Another group of cats were made thrombocytopenic beforehand by treatment with anti platelet serum In such cats infusions of barium sulphate gave no or very weak pulmonary vascular response The presence of a certain number of circulating blood platelets is thus necessary for barium sulphate infusions to have their marked effects on the pulmonary circulation It appears that the essential effect of barium sulphate in this connection is to cause blood platelet alterations with aggregation and probably a subseqent release of vasoactive materials

In a previous publication we have described the occurrence of marked pulmonary vascular and bronchial responses to intravascular platelet aggregation Such aggregation was induced by intravenous administration of collagen (Bo and Hognestad 1972) The responses thus achieved closely resembled those obtained by other workers producing pulmonary embolization with various kinds of material We have proposed therefore that the pulmonary vascular and airway responses to artificial microembolization may depend on the participation of the blood platelets with their aggregation and release of vasoactive substances

The purpose of the present work was to test whether blood platelets really play such a decisive role in this connection Among the many substances employed in experimental microembolization of the lung barium sulphate has been one of the most widely used (Price *et al* 1955 Halmagyi and Colebatch 1961 Nadel Colebatch and Olsen *1946 Clarke Graf and Nadel 1970) It has been clearly shown* that infusion of this material causes marked lung responses Several conclusions concerning the role of reflexes and humoral factors in pulmonary microembolism have been

drawn from these experiments None of the workers using the substance have how ever drawn the attention to the possible role of the platelets in the lung responses described Our conclusion from the experiments to be presented is that barium sulphate intravenously administered does *indeed cause its pulmonary responses* via an interaction with the blood platelets

Some of the results have been presented in a short preliminary communication (Hognestad Bø and Vaage 1972)

## Materials and methods

*Animals* Cats weighing 2 5 to 4 5 kg were used. They were anesthetized by intraperitoneal injections of 30 to 40 mg/kg of sodium pentobarbitone (Nembutal® Abbott)

*Ventilation* After tracheotomy a muscle relaxant Alloferin® (1/2 mg/kg) was given and positive pressure ventilation started with a piston pump respirator (The Ideal Respiration Pump C. F Palmer Ltd London) The respiration frequency was 24 per min With the use of a water seal the end expiratory pressure was kept at 1 cm of water The respirator's tidal volume was adjusted so as to keep the arterial pH at 7 40 Standardized hyperinflations were carefully carried out at regular intervals and between each infusion test

*Surgical procedures Pressure and flow recordings* The thorax was opened widely b a sternum splitting incision. Catheters of polyethylene were introduced into the femoral arteries the pulmonary artery and left atrium for recording for the femoral arterial pressure ($P_{FA}$ with a Statham P23Gb transducer) the pulmonary arterial pressure ($P_{PA}$ with a Statham P23Db transducer) and the left atrial pressure ($P_{LA}$ with a Statham P23De transducer) respectively Infusions were carried out through catheters introduced via the femoral veins and with their tips placed in the caval vein

A flow probe was placed around the ascending aorta, and *aortic flow* was recorded by a Nycotron square wave flowmeter (type 372 Nycotron A/S Norway) The pressure and flow transducers were connected to a six-channel Grass polygraph Model 7 (Grass Instrument Co Mass USA)

The animals were placed on a heated table in order to keep their body temperature at a normal level

*Mean pulmonary vascular resistance* PVR was calculated according to the formula

$$PVR = \frac{P_{PA} - P_{LA}\ (\text{mm Hg})}{\text{Flow (ml/min)}}$$

Percentage changes are given when PVR alterations in different animals are compared

*Thrombocyte counts* in arterial blood samples were carried out according to the method of Brecher and Cronkite (1950)

*pH measurements* were carried out with Radiometer equipment (pH meter 22 equipped with a pH-electrode type G 297/g)

*Barium sulphate infusions* 0 1 g/kg b w of a 30% barium sulphate suspension in saline was given intravenously in the course of 1 min When the subsequent pressor response had vanished (usually after 15 min) a new infusion was given in the same manner This *procedure* was followed in 4 animals Another group of 6 animals was initially also prepared as described above They were then made markedly thrombocytopenic by repeated intravenous infusions of platelet antiserum. At this stage infusions of barium sulphate were carried out

*Platelet antiserum* was prepared as described by Evensen and Elgjo (1972) Citrated blood was obtained by cardiac puncture in anesthetized cats Platelets were separated from the remaining blood corpuscles by differential centrifugation washed 3 times in isotonic sterile saline incorporated in complete Freund's adjuvant and injected i m into a rabbit. This procedure was repeated weekly for 5 weeks The addition of Freund's adjuvant, however was used only for the first injection Each injection contained 2 to $8 \times 10^9$ platelets 10 days after the last injection serum was collected from the rabbit incubated at 56 C for 30 min, absorbed 3 times with packed cat erythrocytes and stored at −20 C (Evensen and Elgjo 1972)

The i v infusions of platelet antiserum themselves gave large pulmonary pressor responses which were very similar to those seen after barium sulphate infusions (Fig 1) When an animal had received 3 infusions of the antiserum a pronounced thrombocytopenia was always achieved and the infusion of barium sulphate could then be given

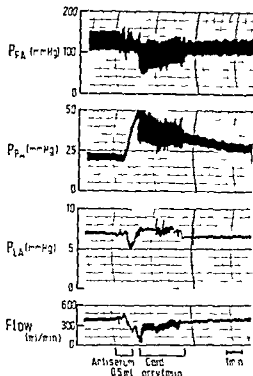



Fig 1

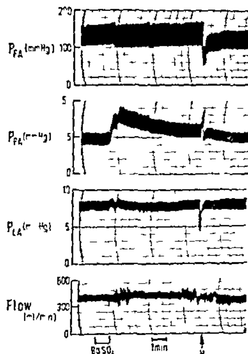



Fig 2

Fig 1 Circulatory effects in a cat of intravenous administration of 0 5 ml anti platelet serum Changes in femoral arterial pressure ($P_{FA}$) pulmonary arterial pressure ($P_{PA}$) left atrial pressure ($P_{LA}$) and in aortic flow (Flow) are shown

Fig 2 Circulatory effects in a cat of intravenous administration of 0 4 ml of a 30 % barium sulphate suspension ($BaSO_4$) Changes in femoral arterial pressure ($P_{FA}$) pulmonary arterial pressure ($P_{PA}$) left atrial pressure ($P_{LA}$) and aortic flow (Flow) are shown At H hyperinflation of the lungs was carried out

*Histological examination of the lungs* The animals were killed by an overdose of Nembutal The fixative (Zenker's solution) was then poured into the bronchial tree so as to prevent collapse of the lungs The lungs were then immediately removed and immersed in the fixative Specimens from all lobes of the lung were embedded and sections 4 μ thick were cut and stained with Haematoxylin Eosin and the MSB method of Lendrum *et al* (1962)

## Results

### *Responses in non treated animals*

Intravenous infusion of the barium sulphate suspension caused a sharp rise in $P_{PA}$ after a delay of about one minute (Fig 2) In the four animals tested there was a mean rise in $P_{PA}$ of 306 % as a response to the first infusion At the same time aortic flow fell in most experiments and on the average by 30 % $P_{FA}$ fell in most experiments whereas $P_{LA}$ showed no change Consequently there occurred a mean rise in PVR of about 490 % but with great variations (range of PVR increase upon first

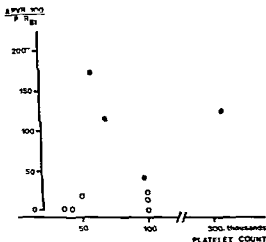



Fig 3 Percentage rise in PVR in two groups of cats upon intravenously administered barium sulphate ($BaSO_4$) ● control animals given 3 previous infusions of barium sulphate ○ thrombocytopenic animals given 3 previous infusions of anti platelet serum. The percentage rise in PVR is related to the level of circulating platelets

infusion 240 % to 700 %) At the peak of the response the level of circulating platelets was reduced to about 50 % of the initial value After 10 to 15 min the pressure and flow values were normalized The number of circulating platelets also showed a rise but never reached the preinfusion level

Further infusions of barium sulphate gave similar but gradually less marked

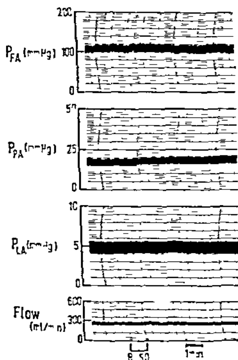



Fig 4 Circulatory effects in a thrombocytopenic cat (< 50 000 blood platelet/mm³ blood) of intravenous administration of 0.5 ml of a 30 % barium sulphate suspension. The animal had been pretreated with anti platelet serum. The circulatory parameters followed are the same as in Fig 1 and 2

TABLE I Mean percentage rise in PVR and mean percentage of circulating blood platelets in 4 animals infused with barium sulphate Each animal received 4 successive infusions the order of which is indicated by roman numbers

| | $\frac{\Delta \text{PVR} \times 100}{\text{PVR}}$ | Level of circulating platelets as per cent of initial value |
|---|---|---|
| | Mean ± SD | Mean ± SD |
| I | 490 ± 203 | 100 ± 0 |
| II | 345 ± 143 | 81 5 ± 2 4 |
| III | 148 ± 103 | 62 5 ± 6 7 |
| IV | 110 ± 53 5 | 62 5 ± 19 8 |

responses At the same time the number of circulating platelets fell in a stepwise manner The correlation between the pulmonary response achieved and the level of blood platelets in arterial blood is seen in Table I

*Responses in thrombocytopenic animals* Six thrombocytopenic animals were given infusions of barium sulphate in the same way as the non treated animals and the responses obtained are given in Fig 3 Three animals showed no lung responses at all (Fig 3 and 4) In the other 3 thrombocytopenic animals only very small responses were obtained Fig 3 also compares the 4th response in animals which had received 3 previous infusions of barium sulphate with the responses in the antiserum treated thrombocytopenic animals

In 3 cats the number of circulating platelets was fairly high after treatment with platelet antiserum Nevertheless only minute responses to barium sulphate infusions were observed in such animals (Fig 3)

*Morphology* In the lungs of animals which had not been made thrombocytopenic beforehand scattered portions of barium sulphate of varying size were found within




Fig 5 Barium sulphate ($BaSO_4$) particles (arrows) surrounded by aggregated platelets lodged in a small artery in the lung

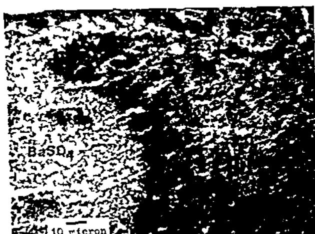



Fig 6 Border area between barium sulphate particles ($BaSO_4$) and platelet aggregates (PA)

the capillaries and other small vessels *Even arteries larger than 200 μm were also* occasionally occluded and then contained barium sulphate The most striking finding however was the accompanying platelet aggregates Fig 5 gives a typical picture The arrows show two portions of barium sulphate located in a small artery Around these areas aggregated blood platelets are seen The vessel lumen is totally occluded by these aggregates which represent the greater part of the space occupying material Fig 6 shows the border area between barium sulphate and aggregated platelets

In the thrombocytopenic group of animals portions of barium sulphate were also occasionally found to occlude vessels but these portions were never surrounded by blood platelets only by leucocytes

## Discussion

The present experiments confirm the findings of other investigators in that i v administration of barium sulphate definitely creates large pressor responses in the pulmonary circulation It was also observed however that the responses to barium sulphate as well as the number of circulating blood platelets decreased with each infusion indicating a platelet-consuming process Trapped aggregated platelets were also found in histological sections from the lungs Such sections clearly revealed the close relationship between the aggregated platelets and the barium sulphate material One can visualize that the whole process might have started with some platelets adhering to the foreign surface of the barium sulphate with a subsequent aggregation of neighboring platelets as a next event When barium sulphate was given to the serum treated thrombocytopenic animals the responses were absent or very weak

The absence of responses to barium sulphate in the thrombocytopenic animals reveals important information First barium sulphate particles do not in themselves trigger any measurable reflex vasoconstriction in the pulmonary vasculature Second

the amount of barium sulphate infused does not give any significant anatomical obstruction of the pulmonary vascular bed. Third, a certain number of normal platelets are needed if barium sulphate is to provoke marked pulmonary responses.

As can be seen from Fig. 3 the level of circulating blood platelets is not the only parameter which influences the size of the responses to barium sulphate. When the reduction in blood platelets has been achieved as a result of previous injections of barium sulphate, higher responses are obtained than when a similar degree of thrombocytopenia has developed from anti-platelet serum infusions. This indicates that the functional state of the platelets must be important in this connection. Anti-platelet serum probably also changes the remaining platelets in such a way that their ability to react with barium sulphate is reduced. It seems probable therefore that release of substances from aggregated platelets directly and/or indirectly gives rise to the observed pressor responses to barium sulphate in the pulmonary circulation.

There is a great similarity between the pulmonary vascular responses obtained upon collagen or antiserum infusions on the one hand, and the present responses obtained with barium sulphate on the other. This similarity in responses again points to a general key role of platelets and platelet changes in many types of pulmonary pressor responses. This is also in accordance with the observations of Thomas and Gurewich (1965) who showed that the level of circulating platelets was of importance for the lethality in rabbits exposed to pulmonary embolization with blood clots of varying sizes.

It has been established that the size of foreign particles used in experimental lung embolization is of great importance for the responses obtained (Niden and Aviado 1956). Why this is so has not yet been satisfactorily explained. With the knowledge that blood platelet aggregation plays such a decisive role, one might suggest that particle size could determine where in the lungs aggregated platelets would be trapped.

Nadel *et al.* (1964) have shown that the pulmonary pressor response to barium sulphate embolism in cats was abolished after depletion of the histamine stores by pretreatment with the histamine releasing agent Compound 48/80. They concluded that barium sulphate is a strong histamine liberator in the lung. We conclude from the present results that barium sulphate must act via the blood platelets which again trigger further events leading to a pulmonary pressor effect. Histamine release might possibly occur in the chain of events started off by the interaction of barium sulphate with blood platelets.

We should also like to stress that when a material is injected for the elicitation of pulmonary microembolization, the possible effects of this material on the blood platelets should always be taken into consideration.

We want to thank dr. Ivar Aursnes for valuable help in preparing platelet antiserum. This work has been supported by a grant to G. Bo from the Norwegian Council on Cardiovascular Research and by a grant to J. Hognestad from Institusjonen Hjelpestikker. It has furthermore been supported through grants to the Institute of Physiology by The Nansen Foundation and by The Norwegian Research Council for Science and the Humanities. All this support is gratefully acknowledged.

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Acta physiol scand 1974 90 252—259

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# Trapping of Radioactive Microspheres in the Pregnant and Non-Pregnant Rabbit

By

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## Abstract

CREASY R K K V KAHANPÄÄ and M DE SWIET *Trapping of radioactive micro spheres in the pregnant and non pregnant rabbit* Acta physiol scand 1974 *90* 252—259

We injected radioactive micro pheres 15 25 and 50 $\mu m$ in diameter in the left ventricle or the lower aorta of rabbits and counted the radioactivity in the lungs to estimate the fraction of microspheres shunted through the systemic circulation. The degree of microsphere shunting was inversely proportional to microsphere diameter The maximum fraction of 15 $\mu m$ microspheres shunted after an aortic injection was 8 8 % as compared with 6 2 % with 25 $\mu m$ spheres and 1 2 % with 50 $\mu m$ spheres There was no statistically significant change in the fraction of microspheres shunted in pregnancy or with anesthesia Shunted microspheres may continue to arrive in the lungs for up to 15 min following aortic injection Micro pheres at least 25 $\mu m$ in diameter should be employed to measure the fractional distribution of cardiac output in rabbits although it may be preferable to use smaller microspheres to measure blood flow within certain organs

One of the fundamental principles of the radioactive microsphere technique for the measurement of regional blood flow is that there should be complete trapping of microspheres in the vascular bed being studied In previous studies of regional blood flow in the rabbit only large microspheres 50 35 and 25 $\mu m$ in diameter have been employed (Neutze *et al* 1968 Duncan 1969 de Swiet and Hoffbrand 1971) Because there are theoretical (Buckberg *et al* 1971) and practical (Phibbs *et al* 1967) advantages in the use of smaller microspheres we planned to use spheres 15 $\mu m$ in diameter to study organ blood flows in the pregant rabbit In preliminary experiments to validate their use we found that a considerable fraction of the 15 $\mu m$ microspheres injected in the left ventricle was trapped in the lungs Also certain organ flows were less than flows reported by other investigators using larger spheres These findings suggested that the 15 $\mu m$ microspheres were not completely trapped

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in the peripheral systemic circulation but were in part shunted to the lungs The present study was designed to investigate the shunting of microspheres 15 25 and 50 $\mu$m diameter in pregnant and non pregnant rabbits Our purpose was to establish the proper size of microspheres to use in measuring regional blood flow in pregnant rabbits and to determine whether pregnancy and its duration had any effect on the magnitude of microsphere shunting

## Material and methods

We used radioactive microspheres labelled with different radionuclides The diameters of the spheres as stated by the manufacturer* were 15 $\pm$ 5 $\mu$m 25 $\pm$ 5 $\mu$m and 50 $\pm$ 10 $\mu$m To determine the exact size of the microspheres we used a micrometer eye piece to examine the diameters of at least 100 microspheres randomly chosen from each batch We then calculated the mean diameter and its standard deviation The suspending liquid of each batch of isotopes was centrifuged and a sample was taken for radioactivity counting

Microspheres were prepared for injection in a saline suspension with a detergent Tween 80 added to prevent aggregation Before each injection the spheres were mixed with an electrical vibrator To achieve high count rates in the lungs and hence high counting accuracy we injected 1 to 6 million counts per minute of each radionuclide This was equivalent to 20 000 to 500 000 spheres depending on the specific activity of the nuclide used for labelling

Non pregnant rabbits and rabbits pregnant 14 to 28 days of the New Zealand white strain weighing 4 to 5 kg were used. Microspheres were injected either into the aorta or the left ventricle Unless otherwise stated the animals were anesthetized with sodium pentobarbital (30 mg/kg iv) and supplementary lidocaine was injected locally For aortic injections a No 3 or 4 French Fogarty embolectomy catheter was inserted into the left femoral artery of the anesthetized rabbit The catheter was advanced so that its tip was 2—4 cm caudal to the left (lower) renal artery A polyvinyl catheter (id 0 76 cm od 0 122 cm) was inserted in the right femoral artery and advanced into the aorta so that its tip was 2 cm below the balloon of the Fogarty catheter

Approximately 1 h later when the blood pressure of the anesthetized animal had become stable we injected a mixture of spheres of two different sizes (various combinations of 15 25 or 50 $\mu$m) labelled with different nuclides Microspheres were injected in 20 s by washing 4 ml of saline through the injector vial attached to the polyvinyl catheter The catheter was then flushed with a further 4 ml of saline injected in 20 s The aortic balloon was inflated just before the aortic injections to prevent reflux flow to the bronchial circulation and then deflated rapidly after the injection The total time of balloon inflation was 60—100 s

For left ventricular injections we placed polyvinyl catheters (id 0 76 cm od 0 122 cm) in the femoral artery at the groin and in the left ventricle via the right carotid artery in anesthetized animals The position of the catheter in the left ventricle was checked by a pressure tracing on the recorder and subsequently by postmortem examination We also made sure that blood could be withdrawn easily from the catheter Both catheters were filled with heparin plugged and led to the base of the ear subcutaneously Two days later injections of microspheres were given through the catheter in the left ventricle

In all experiments the animals were killed with pentobarbital 15 min or more after the last injection of microspheres The catheter positions were checked and the lungs and kidneys removed and carbonized (Rudolph and Heymann 1967) for subsequent radioactivity analysis

All *in vitro* isotope counting was performed with a sodium iodide crystal and a TMC** 100 channel pulse height analysis system

The total counts given to the rabbit were estimated by counting the injection vial before and after each injection The high count rate of the injectate was corrected for coincidence losses by constants derived previously for our equipment in our laboratory The pulse height analysis system was then used to separate the different nuclides The count rates in the carbonized lungs and kidneys were corrected for differences between the counting geometry of spheres distributed throughout carbonized tissue and the geometry of spheres in the injected settled at the bottom of the injector vial The fraction of spheres shunted through the systemic circulation was calculated by dividing the lung counts by the total counts given to the rabbit and was expressed as a percentage

* Minnesota Mining and Manufacturing Co St Paul Minnesota U.S.A

** Technical Measurement Corporation North Haven Conn

TABLE I. Size of Microspheres

| Isotope | Given Diameter μm | Observed Diameter μm | Small Spheres* | Large Spheres+ |
|---|---|---|---|---|
| $^{141}$Ce | 15 | 15 (2.7) | 0 | 1 |
| $^{51}$Cr | 15 | 17 (3.2) | 0 | 12 |
| $^{85}$Sr | | | | |
| Batch 1 | 15 | 15 (2.1) | 1 | 0 |
| Batch 2 | 15 | 14 (3.9) | 1 | 7 |
| $^{95}$Nb | 15 | 18 (2.1) | 0 | 7 |
| $^{141}$Ce | 25 | 28 (2.7) | 1 | 1 |
| $^{85}$Sr | 25 | 25 (4.8) | 3 | 0 |
| $^{125}$I | 50 | 59 (8.1) | 4 | 0 |

Numbers in parentheses indicate the standard deviation of the mean.
* < 9 μm for 15 μm spheres, < 17 μm for 25 μm spheres, < 35 μm for 50 μm spheres.
+ > 21 μm for 15 μm spheres, > 33 μm for 25 μm spheres, > 65 μm for 50 μm spheres.

## Results

The size distribution of each batch of microspheres is presented in Table I. The estimated diameter of the spheres varied from the stated diameter as follows: in the 15 μm batches, the maximum variation of the mean was 3 μm (range 14—18); in the 25 μm batches, 3 μm (range 25—28); and in the 50 μm batches 9 μm (range 45—59). Also, only few small spheres were found in each batch since the size distribution was skewed so that most of the variability of the mean occurred among the large microspheres of each batch (see Table I). The fraction of radioactivity free in the supernatant was always less than 0.25 %.

The time course of microsphere shunting was established by injecting microspheres into the aorta of three 28 day pregnant rabbits. The radioactivity count rate in the lungs was determined with a counter which had a 2.5 cm highly collimated opening to the crystal (Nuclear Chicago) placed against the chest wall. The output of the counter was analysed by a rate meter (Nuclear Chicago) and displayed on a Beckman recorder. When the injectate was placed in a syringe over the pelvis of the rabbit, the external counter only recorded 10 % of the final lung count after injecting microspheres. This indicates that the collimation was efficient and that about 90 % of the counts finally recorded by the external counter came from the lungs. In the three rabbits the count rate in the lungs continued to increase for up to 10—15 min after microsphere injection (Fig. 1), indicating that the time of transit of microspheres from the aorta through the peripheral circulation to the lungs could be as long as 15 min. Therefore, in all studies, we allowed an interval of at least 15 min after the last injection of microspheres before killing the animal.

Preliminary studies to evaluate our aortic injection procedure were performed on three groups of 28 day pregnant rabbits.

First, to determine whether pentobarbital anesthesia affected microsphere shunting, we injected 15 μm and 25 μm spheres separately into the aorta of four pregnant rabbits that had received local lignocaine anesthesia only. The mean fraction of 15

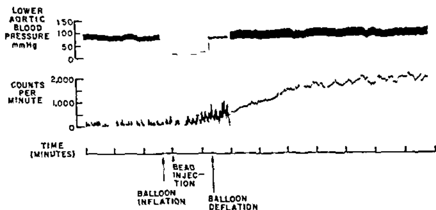



Fig 1 Time course of microsphere shunting
*Upper trace* blood pressure measured in the lower aorta occluded at the time of balloon inflation
*Lower trace* count rate in the lungs following microsphere injection in the lower aorta (see text) The count rate from the lungs continued to increase indicating the arrival of shunted microspheres in the lungs for 7 min after microsphere injection

μm spheres shunted was 1 77 % (SD 1 71 range 0 90—5 17) and that of 25 μm spheres 1 70 % (SD 3 77 range 0 19—9 57) The fraction of 15 μm and 25 μm microspheres shunted in these conscious animals did not vary significantly (P > 0 5) from the fractions of 15 μm and 25 μm microspheres shunted in subsequent studies on 28 day pregnant animals anesthetized with pentobarbital (group C Table II)

To determine whether the ischemia produced by balloon inflation had an effect we studied a group of seven anesthetized animals We prepared the animals as for aortic injections except that the tip of the injection catheter was placed lower just above the aortic bifurcation Each animal received an injection of 15 μm or 25 μm microspheres or a mixture of the two sizes separately labelled slowly over a 2 min period without balloon inflation A second similar injection was given 15 min later with the balloon inflated The systemic arterial pressure of the animals did not vary between the two injections In nine observations on seven animals balloon inflation had no apparent effect on shunting Despite considerable individual random variation the shunting of microspheres after the first and second injections did not differ systematically (Fig 2 A)

We investigated the variability of microsphere shunting further in a group of five anesthetized animals Two injections of 15 μm microspheres were given in succession to each animal under the same experimental conditions with the balloon inflated The results showed considerable random variation in the fractions of microspheres shunted as well as individual variations between animals (Fig 2 B)

We then determined the magnitude of shunting in a series of studies on seven groups of non pregnant animals after intra aortic and left ventricular injection of 15 25 and 50 μm microspheres The results of our 96 observations on 46 animals are summarised in Table II Because the degree of shunting was often not normally distributed about the mean we have shown the range as well as the standard devi

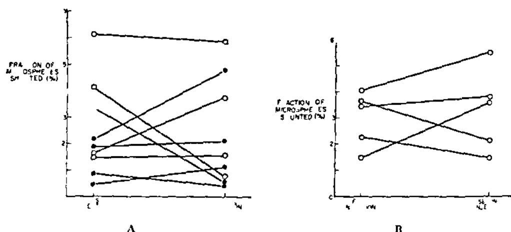



Fig 2 *A Effect of balloon inflation and ischemia on microsphere shunting* Microspheres 15 µm in diameter (○) and 25 µm in diameter (●) were injected in the lower aorta without balloon inflation (first injection) and then with balloon inflation (second injection) There was no systematic difference in the fraction of microspheres shunted

Fig 2 *B Variability of microsphere shunting* Two successive injections of 15 µm microspheres were given in the lower aorta with balloon inflation There was considerable random variation both between animals and within animals in the fraction of microspheres shunted

TABLE II Fraction of 15 µm 25 µm and 50 µm microspheres found in the lungs after injection into the aorta or left ventricle of nonpregnant and pregnant rabbits

| | Microspheres Shunted 15 µm | 25 µm | 50 µm |
|---|---|---|---|
| *Aortic Injection (anesthesia)* | | | |
| Group A (nonpregnant) | | | |
| Mean ± S D | 2 80 ± 2 70 (9) | 1 65 ± 1 34 (9) | 0 51 ± 0 46 (5) |
| Range | 0 56 ± 8 79 | 0 16 ± 3 70 | 0 06 – 1 17 |
| Group B (14 day pregnant) | | | |
| Mean ± S D | 2 98 ± 1 57 (6) | 0 84 ± 1 57 (5) | |
| Range | 1 64 – 5 90 | 0 36 – 1 28 | |
| Group C (28 day pregnant) | | | |
| Mean ± S D | 3 49 ± 2 15 (5) | 1 97 ± 2 47 (5) | 0 10 ± 0 05 (5) |
| Range | 1 45 7 05 | 0 31 – 6 91 | 0 07 – 0 17 |
| *Left Ventricular Injection (no anesthesia)* | | | |
| Group D (nonpregnant) | | | |
| Mean ± S D | 5 56 ± 3 33 (15) | | |
| Range | 1 90 – 13 20 | | |
| Group E (nonpregnant) | | | |
| Mean ± S D | | 4 31 ± 1 95 (10) | |
| Range | | 2 01 – 8 47 | |
| Group F (28 day pregnant) | | | |
| Mean ± S D | 10 20 ± 2 20 (12) | | |
| Range | 6 40 – 13 10 | | |
| Group G (28 day pregnant) | | | |
| Mean ± S D | | 6 22 ± 1 46 (10) | |
| Range | | 3 95 – 9 54 | |

Number of observations is given in parentheses

ation. Pregnancy had no statistically significant effect on the fraction of micro spheres shunted and furthermore we could not demonstrate any apparent difference between the fractions of microspheres shunted at 14 days and 28 days gestation (groups B and C). In both gestational groups as well as in the non pregnant animals the degree of shunting was inversely proportional to the sphere size as shown Table II. These differences in the shunting of 15 25 and 50 μm micro spheres however were not statistically significant because of the variability.

The lung counts after left ventricular injections of 15 μm spheres were significantly higher ($P < 0.01$) than the lung counts after aortic injections presumably because of bronchial circulation as well as the different vascular bed being supplied. After aortic injections the radioactivity in the kidneys was always less than 1 % of that found in the lungs in each animal. No radioactivity was found in the fetuses of the pregnant animals.

## Discussion

In studies with radioactive microspheres the radioactivity in the lungs can be used as an indicator of the trapping efficiency of the systemic vascular bed. Ring *et al* (1961) injected non radioactive polystyrene microspheres in the pulmonary artery of the dogs and showed that very few microspheres bigger than 15 μm in diameter were found in the systemic circulation. Their findings were based on semi-quantitative microscopic observations. Furthermore studies in rabbits (Gordon *et al* 1953) and in dogs (Kaihara *et al* 1968) have shown that the pulmonary vascular bed traps nearly all particles larger than 15 μm diameter. In our studies the very low radio activity counts in the kidneys relative to the high lung counts after low aortic injec tions is also evidence of the good trapping efficiency of the lung.

The amount of radioactivity in the lungs can be detected *in vivo* by external counters or *in vitro* by direct counting after the animal has been killed and dis sected. We used both methods. By external counting we were able to demonstrate that the shunting of microspheres may continue for up to 10 to 15 min after left ven tricular injection. For this reason the estimation or exclusion of organ shunts by venous sampling is inaccurate since it is seldom practical to collect venous blood from an organ for as long as 15 min.

Prinzmetal et al (1948) first studied microsphere shunting in rabbits using glass spheres 60—180 μm in diameter and found that they could cross the liver from the portal vein to the lung and that they also crossed the spleen from the splenic artery to the liver. However these studies were not quantitative. Neutze *et al* (1968) in jected 50 μm microspheres into the lower aorta of rabbits and found that 0.03 % to 0.25 % passed to the lung. Only a very small portion of the total radioactivity was found in the lungs by Hoffbrand *et al* (1969) in conscious rhesus monkeys (0.5 %) or by Kaihara *et al* (1968) in conscious dogs (1.7 %) after left ventricular injection. Both investigation groups used 50 μm spheres. These values are comparable with our mean value of 0.5 % for 50 μm microspheres found in lungs of the non pregnant

female rabbits Duncan (1969) injected 50, 35 and 25 μm microspheres into the left ventricle of pregnant rabbits. She too noted that a very small amount of each diameter microsphere was found in the lungs but did not give precise quantitative data.

Kaihara *et al.* have also studied 15 μm microspheres. They found that 5 to 10 % of spheres injected into the systemic circulation of conscious dogs was trapped in the lungs.

The amount of microsphere shunting during physiological conditions does not only vary according to the sphere size but it also seems to be different in different vascular beds.

Delaney (1964; Delaney and Grim 1969) observed that 4.5 % to 29 % of 20 μm microspheres injected into the dog femoral artery was eventually trapped in the lungs. Thus the vascular bed supplied by the femoral artery may well be the source of the very high fraction of microspheres found to be shunted by Kaihara *et al.* (1968). In contrast, there seems to be little shunting across the heart or kidney. Buckberg *et al.* (1971) showed that less than 1 % of 8—10 μm microspheres were shunted across the coronary circulation of the dog. McNay and Abe (1970) withdrew blood from the renal vein during microsphere injection in the dog and found that less than 0.5 % of 19, 27 and 35 μm microspheres was shunted across the kidneys. Slotkoff *et al.* (1971) also collected from the renal veins in the dog and found that less than 0.2 % of 16.8 μm microspheres was shunted.

It has been reported that anesthesia may affect microsphere shunting although pentobarbital had no effect in our rabbits. In contrast, Kaihara *et al.* (1968) found that 17 % of 15 μm microspheres injected into the aorta was shunted to the lung in anesthetized dogs compared with 5—10 % in conscious animals. However, Delaney (1969) found that the fraction of microspheres shunted from injection in the superior mesentery artery in the dog decreased with pentobarbital anesthesia. Increased blood flow to the lungs during pentobarbital anesthesia has been observed by Hoffbrand *et al.* (1969) in monkeys and recently by Sasaki *et al.* (1971) in rats with 50 μm spheres. These investigators point out that this could in part have been due to an increase in arteriovenous shunting of microspheres through the systemic circulation under anesthesia in these species.

In the sheep, Makowski *et al.* (1968) found that less than 1.5 % of 25, 35 and 50 μm microspheres injected into the aorta was shunted acros the uterus. Lees *et al.* (1971) observed a similar figure for 50 μm microspheres shunted which increased to 2.2 % in pregnancy. However, this difference was not statistically significant.

There was no statistically significant difference in shunting of microspheres in our pregnant rabbits compared with the non-pregnant animals although there was a trend to an increased shunting among the conscious pregnant animals studied with 15 μm microspheres. Our results, however, do not disprove the existence of physiological shunts in the pregnant rabbit uterus.

In conclusion, it would seem that there is very little shunting of microspheres 25 μm in diameter or larger in rabbits, sheep, monkeys or rats. There may be, however,

considerable shunting of microspheres less han 25 μm in diameter in dogs and as our results demonstrate in rabbits

We would therefore recommend the use of microspheres at least 25 μm in diameter for the measurement of the fractional distribution of cardiac output in non pregnant and pregnant rabbits If it is necessary to use smaller microspheres for the measure ment of blood flow within organs the completeness of trapping of microspheres should be checked for each organ

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Acta physiol scand 1974 90 260–266

From the Zoophysiological Laboratory Department of Zoology University of Turku Finland

# Lowered Chronotropic Sensitivity of Rat and Frog Hearts to Sympathomimetic Amines Following Cold Acclimation

By

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## Abstract

Tirri R M N J Harri and L Laitinen *Lowered chronotropic sensitivity of rat and frog hearts to sympathomimetic amines following cold acclimation* Acta physiol scand 1974 90 260–266

Cold acclimation elevated the basic heart rate in the rat and decreased the chronotropic sensitivity to noradrenaline and isoprenaline. These changes reached their maximum after a week but returned to the initial level after a month of prolonged cold acclimation. Phenylephrine failed to increase the heart rate under the *in vivo* conditions used. In the frog cold acclimation lowered the heart chronotropic sensitivity most significantly to phenylephrine, less to adrenaline and not at all to isoprenaline, as indicated by changes in the $ED_{50}$ values. This lowered sensitivity to adrenaline reached its maximum level after 2–4 days of cold exposure and had not returned to normal after 20 days. In both cold- and warm-acclimated frogs the elevation of the temperature where the heart rate was measured decreased the sensitivity to phenylephrine.

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It has been shown that cold acclimation leads to a gradual increase in the metabolic response to noradrenaline (NA) in mammals (Hsieh and Carlson 1957, Depocas 1960, Himms-Hagen 1969) and to adrenaline (A) in amphibians (Harri and Hedenstam 1972). There are, however, controversial reports about the pressor and cardiac responses to NA in cold acclimated animals: the response to NA may be increased (LeBlanc 1960, Heroux 1961, Lvonuk and Hannon 1963), or unchanged (Himms-Hagen and Mazurkiewicz-Kwilecki 1970), or variable according to the measuring temperature (Reite *et al* 1966). The aim of the present study was to compare the possible changes in the cardiac chronotropic sensitivity to sympathomimetic amines in the course of cold acclimation in the rat with those in the frog. In order to prevent the influence of vagal reflexes the responses were recorded from denervated hearts.

Some recent studies have shown that a change of the measuring temperature affects the metabolic rate of isolated heart tissue and thereby also its sensitivity to sympathomimetics in mammals (Oppermann *et al* 1969, 1971, 1972, Reinhardt *et al* 1972, Schumann *et al* 1972) and in amphibians (Kunos and Szentiványi 1968

Buckley and Jordan 1970 Harri 1973) Since temperature acclimation is also known to alter the metabolic rate it was of interest to compare the effect of cold acclimation with the effect of different measuring temperatures on the heart sensitivity to sympathomimetic drugs in frog

## Material and methods

Adult male Sprague Dawley rats weighing on an average 326 g (range 213—470 g) were acclimated to 3 C for 1 2 4 7 and 30 days The control animals were kept at 23 C The rats were anesthetized with 50 mg/kg of pentobarbital s c and the n vagi and the sympathetic nerve trunks were cut Respiration was maintained artificially The heart rate was recorded by a Mingograph 24B jet recorder with chest location of the leads The chronotropic effects of adrenergic agonists NA isoprenalin (ISO) and phenylephrine (PHE) were recorded after injecting them into the femoral vein in 0 1 ml of saline Only 1 to 2 injections were given to each rat in order to avoid the tachyphylactic and other side effects The heart rate recordings lasted for 5 5 min after the injections and the time interval between two successive injections was 8 5 min

Adult winter frogs (*Rana temporaria*) of both sexes weighing 30—40 g were acclimated to 25 C for at least 2 weeks before use (warm acclimated frogs) and then transferred to 5 C for 1 2 4 10 and 20 days (cold acclimated frogs) The animals were pithed The drugs were injected into the abdominal vein via polyethylene tubing and the dose response curves for the chronotropic responses to the drugs used were determined as described earlier (Harri 1973)

The following drugs were used pentobarbital sodium (mebumalum NFN) (Nembutal® Abbott) l noradrenaline D hydrogentartrate (Fluka AG) (NA) l adrenaline base (Merck AG) (A) isoprenaline hydrochloride (Isuprel® Winthrop) (ISO) phenylephrine hydrochloride (metaoxedrinum NFN) (Neo-synephrine® Winthrop) (PHE) The doses are expressed in terms of free drug The temperature of the solutions injected was that of the animal

## Results

*Rats* The basic heart rate measured from the denervated hearts was 336 ± 2 5 beats/min (N = 152) in the control rats After transferring them to 3° C the heart rate was slightly elevated reaching its maximum (348 ± 2 7 beats/min N = 135) in 7 days after the transfer (P < 0 005 when compared with the control animals) and then returned to the initial level (336 ± 5 2 beats/min N = 48) after 30 days of cold exposure When injected into the femoral vein NA and ISO elicited marked tachycardia The maximum effects were reached in 30 s Then the heart rate gradually slowed down to the initial level in 3 to 5 min after NA injection and in 5 to 10 min after ISO Fig 1 gives the results obtained at 30 s after the injections It can be seen that the chronotropic responses to NA were significantly smaller in rats acclimated at 3° C for 7 days than in the control animals (P < 0 05 with 0 5 μg/kg of NA P < 0 01 with 2 5 μg/kg of NA and P < 0 025 with 5 0 μg/kg of NA 5—13 animals in each group) Because the effect of NA did not become any stronger with doses exceeding 12 5 μg/kg the combined result from doses of 12 5 25 0 62 5 125 and 167 μg/kg is given in the graph This maximum response was 25 6 ± 0 74 % (N = 13) in control rats but only 15 3 ± 1 13 % (N = 5) in animals acclimated to cold for a week These values differ significantly (P < 0 001) from each other indicating that cold acclimation also decreased the maximum response to NA Because the basic heart rate was only 3 5 % higher in these animals than in the controls the absolute values for the heart rates after the administration of NA were markedly lower in cold acclimated rats as well After the rats had been at 3° C for

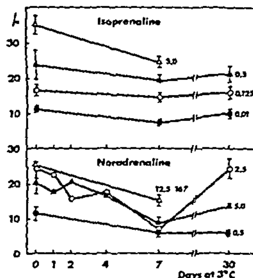



Fig 1 The precentage increases in the heart rates caused by different doses of noradrenaline and isoprenaline in 23 C-acclimated control rats and in animals transferred to 3 C for different lengths of time The doses used are marked at each line (in μg/kg) The mean responses ± S E are given

30 days the response to NA had returned to the values of the control animals

Correspondingly the acceleration in the heart rate caused by ISO was significantly smaller in the rats acclimated at 3 C for 7 days than in the controls with a dose of 0 01 μg/kg ($P < 0\ 005$ N = 23+22) and also with a dose of 5 0 μg/kg ($P < 0\ 01$ N = 6+5) which gave the maximum response to ISO As with NA the responses to ISO in rats acclimated at 3 C for 30 days no longer differed from those in control animals

PHE with a dose of 2 5 μg/kg elevated the heart rate by 2 0 % in the control rats and by 0 67 % and 1 09 % in the rats acclimated at 3 C for 7 and 30 days respectively The corresponding values with a dose of 10 μg/kg were 1 43 % in the controls and 2 20 % and 3 02 % in the 7 and 30-days cold acclimated rats respectively These responses do not differ significantly from the basic heart rates in any group

Since the subsensitivity to NA as a result of a weeks cold acclimation was accompanied by the subsensitivity to ISO as well and since PHE failed to increase the heart rate it can be concluded that in the rat this phenomenon is mediated by the β adrenoreceptors

*Frogs* From Fig 2 it can be seen that in the warm acclimated frogs the basic heart rates were significantly higher ($P < 0\ 02$) when measured at 25 C and slightly higher still when measured at 12° C (N S ) When injected into the abdominal vein the drugs used elicited their effects on the heart rate in a few seconds and the effect lasted unchanged for at least 5 min even at the higher measuring temperature Fig 2 shows the heart rates during the 30 to 90 s post injection period (for further details see Harri 1973 The maximum responses to the drugs tended to be higher in cold acclimated frogs However these values do not differ significantly from the warm acclimated groups in any of the cases studied

Fig 2 shows further that both temperature acclimation and a change in the

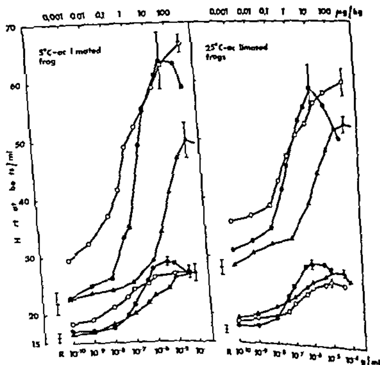



Fig 2 The log dose response curves for the chronotropic responses to adrenaline (dots) isoprenaline (open circles) and phenylephrine (triangles in 5 C and 25 C-acclimated frogs when measured at 25 C (upper curves) and at 12 C (lower curves) R = the basic heart rates after the injection of Ringer only Each curve represents the mean of 5 to 6 animals The basic heart rates and the maximum responses are represented with vertical bars indicating ± S E The concentration of drugs in the injection fluid (in g/ml) and the doses in terms of µg/kg are given

TABLE I Effect of temperature acclimation and measuring temperature on the $ED_{50}$ values for positive chronotropic responses to sympathomimetic amines in the frog 5 to 6 animals in each group

| Drug | Acclimation temperature C | ED measured at 25 C ng/kg (Mean ± S E ) | Acclimation effect at 25 C* | ED measured at 12 C, ng/kg (Mean ± S E ) | Acclimation effect at 12 C* | $ED_{50}$ measured at 25 C / ED measured at 12 C |
|---|---|---|---|---|---|---|
| Isoprenaline | 5 | 223 ± 66 | 1 4 | 311 ± 102 | | 0 77 |
| | 25 | 156 ± 31 | | 344 ± 56 | 0 9 | 0 45 |
| Adrenaline | 5 | 880 ± 168 | 3 0 | 628 ± 97 | | 1 40 |
| | 25 | 291 ± 45 | | 01 ± 49 | 3 1 | 1 4 |
| Phenylephrine | 5 | 23200 ± 6650 | 3 8 | 2,60 ± 387 | | 3 |
| | 25 | 6190 ± 1860 | | 912 ± 27 | 13 0 ** | 3 |

ED of 5 C-acclimated frogs/ED of 25 C-acclimated frogs
Statistical significances of the differences of ED values between 5 C and 25 C and between ED values measured at 25 C and at 12 C P < 0 02 P < 0 01 and 0 001

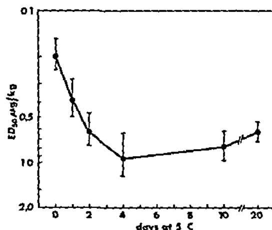



Fig. 3. The course of time for the decrease of chronotropic sensitivity (= increase in the $ED_{50}$-values) to adrenaline in frogs acclimated to 25° C and then transferred to 5° C when measured at 12° C. Each point represents the mean of 5 to 6 animals ± S.E.

measuring temperature caused a shift in the dose response curves for an individual drug. The extent of these shifts is shown in Table I as the changes in the $ED_{50}$ values. The results show that cold acclimation decreased the chronotropic sensitivity of the heart to PHE by 3.8 times when measured at 25° C and by 13.0 times when measured at 12° C, as indicated in the $ED_{50}$ ratios of 5° C acclimated frogs to 25° C-acclimated frogs. On the other hand, the sensitivity to ISO did not change according to temperature acclimation. The results with A were intermediate between these. This probably indicates that cold acclimation caused subsensitivity in the cardiac α adrenoreceptors. Correspondingly, an increase of the measuring temperature from 12° C to 25° C resulted in the most marked decrease in the sensitivity to PHE both in warm- and cold-acclimated frogs. In warm-acclimated animals, on the other hand, the sensitivity to ISO was significantly ($P < 0.02$) higher when measured at 25° C. The results indicate that an elevation of the measuring temperature lowered the sensitivity of the cardial α receptors but slightly increased the sensitivity of β receptors.

The results in Fig. 3 show that the sensitivity of the frog's heart to A decreased very rapidly after transferring the warm-acclimated animals to 5° C. The $ED_{50}$ values typical for frogs acclimated to 5° C were observable already 2 days after the exposure ($P < 0.02$). Later changes were not significant.

## Discussion

The results show that the lowered sensitivity to sympathomimetic amines seems to be a common feature for the cold-acclimated rat and frog. However, there are also differences between these. The period of time for the development of maximum subsensitivity was shorter in the frog. Furthermore, in contrast to the frog, this phenomenon was temporary in the rat. In the latter group cold acclimation also elevated the basic heart rate and decreased the extent of maximum responses to NA and ISO, whereas in the frog the basic heart rate tended to be lower and the

maximum responses larger to all the drugs studied in cold acclimated animals The most striking difference between rat and frog was however the different type of adrenoreceptor in the rat the cold acclimation subsensitivity seems to be mediated by $\beta$ recptors but in the frog by $\alpha$ receptors It is interesting to note that a similar difference between homeothermic and poikilothermic vertebrates is also found in the type of adrenoreceptors influenced by temperature alteration a decrease in the measuring temperature leads to a chronotropic and inotropic supersensitivity of $\beta$ receptors in mammals (Oppermann *et al* 1971 Reinhardt *et al* 1972 Schumann *et al* 1972) but to that of $\alpha$ receptors in amphibians (Kunos and Szentivanyi 1968 Buckley and Jordan 1970 Harri 1973)

The lowered chronotropic ensitivity to adrenergic drugs in cold acclimated animals is very significant as a physiological adaptation phenomenon since it protects the animals from excessive tachycardic effects from NA (or A in amphibians) released as a result of low temperatures and reduces the necessity of vagal reflexes

Recently it has been found that in the guinea pig atria an elevated metabolic rate desensitizes the $\beta$ receptors (Reinhardt *et al* 1972 Schümann *et al* 1972) Thus the elevated metabolic rate in the heart from cold acclimated rats might be responsible for the results observed This is supported by the results observed in the frog In this animal cold acclimation induced a lowered chronotropic ensitivity to PHE rather similar to that probably induced by the elevated metabolic rate at the higher measuring temperature On the other hand an alteration of the measuring temperature did not significantly change the sensitivity to A while cold acclimation decreased it very clearly Furthermore in contrast to temperature alteration cold acclimation did not change the sensitivity to ISO These results together with some earlier observations (Kunos and Szentivanyi 1968 Buckley and Jordan 1970 Harri 1973) support the view that the altered metabolic rate may accompany but probably is not the only reason for the sensitivity changes observed as a result of cold acclimation

It is known that a prevention of the release of NA from its peripheral stores leads to a supersensitivity similar to that of decentralization (Trendelenburg 1963) It could be assumed that the increased release of NA in cold acclimation rats might lead to the opposite results *i e* to those observed in this study However the release of NA in cold exposed rats decreases only by a little in one month (Leduc 1961) while the subsensitivity to this amine is already over within this time Thus if the increa ed release of NA desensitizes the receptors the sensitivity will return although the higher NA release still continues

Furthermore it is possible that in addition to the possible direct effect on adrenergic receptors the increased release of NA (or A in amphibians) may also influence the metabolic rate of the heart tissue and thereby indirectly change its sensitivity to adrenergic drugs

On the other hand Oppermann *et al* (1972) report that an increase in catechol O methyl transferase (COMT) activity is related to a lowered sensitivity of guinea pig heart to catecholamines as a result of elevated measuring temperature It is well known that the activity of several enzymes changes simultaneously with a change in

metabolic rate Therefore it is po sible that COMT activity is also incre ed in hearts of cold acclimated animals with higher metabolic rate and this is the reason for lowered ensitivity to sympathomimetic amines observed in this study This pos sibility is however unsatisfactory as an explanation for the results obtained in the frog since in this animal the chronotropic sensitivity to ISO did not change by cold acclimation in spite of the fact that also this substance is a substrate for COMT Moreover the fact that there are also results which contradict to this study (Heroux 1961 Evonuk and Hannon 1963) indicates that he mechanism(s) by which cold ac climation changes the chronotropic sensitivity to sympathomimetic amines may be very complex in nature and that further experiments are needed before it can be elucidated

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From the Department of Anatomy University of Helsinki Finland

# Observations on Closed Tissue Cultures of Sympathetic Ganglia of Chick Embryos in Media Buffered with N-Tris-(Hydroxymethyl) Methyl Glycine or N-2-Hydroxy-Ethyl-piperazine-N-2-Ethanesulfonic Acid

By

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## Abstract

HERVONEN H and L RECHARDT *Observations on closed tissue cultures of sympathetic ganglia of chick embryos in media buffered with N tris(hydroxymethyl) methyl glycine or N 2 hydroxy ethylpipera ine N 2 ethanesulfonic acid* Acta physiol scand 1974 *90* 267—277

A simple and practical closed tissue culture system for sympathetic ganglia is described It has pro ed suitable in metabol c and pharmacological studies of sympathetic neurons where it is necessary to ma ntain the neurons in good condition for longer periods The effects of coverslip Nerve Growth Factor (NGF) and various concentrations of glucose and serum on nerve fibre growth were estimated in N tris(hydroxymethyl)methyl-glycine (TRICINE) or N 2 hydroxy ethylpiperazine N 2-ethanesulfonic acid (HEPES) buffered culture mediums TRICINE and HEPES buffers were found non toxic to the nervous tissue and by these buffers it was possible to avoid $CO_2$-gassing and to use a closed environment which facilitated daily checking of ex plants

Although the sympathicoblasts were not dissociated before culturing a monolayer of the neurons with dense network of nerve fibres formed in this culture system. This pattern of growth made it possible to perform histochemical reactions (fluorescence microscopy and electron microscopical histochemical reactions) directly in the culture dishes

Tissue culture constitutes an ideal model of *in vitro* conditions for studying the development and metabolism of sympathetic neurons Although so far several culture methods for sympathetic neurons have been described see *e g* Burdman 1968 Eränkö *et al* 1972 Kim and Munkacsi 1972 Larrabee 1970 Lever and Presley 1971 Levi Montalcini and Angeletti 1968 Sano *et al* 1967 they are methodologically complex and usually require an artificial atmosphere containing CO

The aim of the present study was to develop a tissue culture method which would fulfil the following requirements (1) to maintain the sympathetic ganglion cells of chick embryo living and in good condition for 1—2 weeks (2) to be po sible to perform histochemical reactions directly in the culture chambers on the intact explants for studying the neurotransmitter metabolism (3) to reach an appropriate

accuracy in the metabolic and pharmacological experiments i e the proportion of the natural media serums and embryo extracts in the final culture medium should be minimized (4) the troublesome CO gassing should be avoided, (5) the interval for changing the culture medium should be at least one week and (6) the daily checking of the explants by phase contrast microscopy should be possible

The importance and critical evaluation of several factors essential to sympathetic neuronal growth were considered including (1) the possible preference of micro milieu for growth (2) the suitability of new buffers TRICINE and HEPES (Darzynkiewicz and Jacobson 1971, Gardner 1969 Good *et al* 1966) for tissue culture of nervous tissue and (3) the effects of different pH values the concentration of glucose Nerve Growth Factor (NGF) (see *e g* Levi Montalcini and Angeletti 1972) and serum on the growth and viability of the neurons

## Material and Methods

The lumbar sympathetic ganglia were dissected from 12 day old chick embryos of the white Leghorn strain The fertile eggs were incubated for 12 days at 37 C with 60 relative humidity The lumbar sympathetic truncs were first separated and the individual ganglia then dissected by cutting the truncs between each ganglia Free hand dissections were carried out with thin forceps and glass instruments in culture medium under the dissecting microscope *The individual ganglia were placed on surface treated plastic petri dishes (C A Greiner et Sohne Nurtingen Germany)* 35 mm of diameter containing 1 ml of the final culture medium Two or three ganglia were cultured in each petri dish To avoid excessive evaporation the dishes were hermetically sealed with paraffin wax

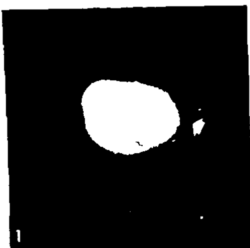
In micro-milieu studies the ganglia were first placed on a petri dish in a drop of the culture medium Glass coverslips (Chance Bros Birmingham England or Clay Adams Inc New York USA) were then pressed on the drop and anchored with silicone grease (Silicon High Vacuum Grease Edwards High Vacuum Ltd Crawley Sussex England) Thereafter the main culture medium was added and the dishes were sealed as described above All the procedures were carried out aseptically The dishes were kept in a culture room at a temperature of 37 C (±0 5 C) The cultures were maintained for 1—2 weeks some of them up to 2 months

The culture medium was based on Parker s Medium 199 (Manufactured by GIBCO and dissolved in distilled water by Orion Helsinki Finland) N 2 hydroxy ethylpiperazine N 2 ethane sulfonic acid (HEPES) and N tris(hydroxymethyl)methyl glycine (TRICINE) (*both manufactured by Sigma St Louis Miss USA) were used as buffers in the preliminary studies* TRICINE was selected for further use and was employed in all test series as a buffer It was prepared as follows TRICINE powder was dissolved in distilled water to make an 1 M solution and the pH was adjusted with 1 N NaOH Five ml of the stock solution was added to 100 ml of the culture medium to achieve a 0 05M TRICINE concentration in the final culture medium

In the pH serie the effects of pH 7 0 7 1 7 2 7 3 and 7 4 of the final culture medium in 36 C temperature were tested Heat inactivated newborn calf serum (Orion Helsinki) was added to make 5 10 and 20 concentrations In some experiments it was omitted The glucose concentrations of 0 85 g/l 1 5 g/l 3 0 g/l and 6 0 g/l were reached by adding respective amounts of 0 2 M aqueous glucose solution

Purified and standardized NGF from Burroughs and Wellcome (England) was added to the medium immediately before culture preparation NGF was used in a concentration of 1 IU/ml and 2 IU/ml Some cultures were tried without NGF G penicillin 100 IU/ml was used as an antibiotic in all media All solutions were sterilized by filtration through Millipore filters pore size 0 22 nm

The observation on fibre growth were made after 12 24 and 48 h and 3 4 7 10 and 14 days using a Wild phase contrast microscope The fibre growth was measured with an ocular grid in 2 vertical and horizontal directions between the border of the implanted ganglia and the farthest point where a distinct network still existed between the fibres The means were estimated from these values which were handled statistically by using Student s t test Altogether over 700 cultures were made and in each experimental test series 30 ganglions were measured







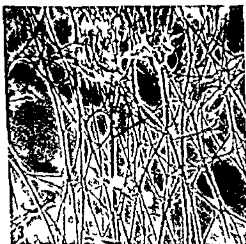



Fig 1 Sympathetic ganglion cultured for 12 h in TRICINE buffered Parker s Medium 199 pH 7.3 consisting of 3 0 g/l glucose 10% calf serum 1 IU/ml NGF and 100 IU/ml G-penicillin A dense anastomosing network of thin nerve fibres (arrow) fills the space between two thicker nerve trunks Growth has begun asymmetrically Dark field microscope × 60

Fig 2 2 explants of sympathetic ganglia cultured for 4 days Culture medium as in Fig 1 Richly anastomosing connections appear between the two ganglia. Dark field microcsope × 60

Fig 3 Sympathetic ganglion cultured for 7 days Culture medium as in Fig 1 A view of nerve fibres from the middle area of the fibre halo Some fibroblasts are seen under the fibres (arrows) Phase contrast microscope × 150

## Results

*Equipment* Best results were obtained by using strictly disposable instruments and dishes throughout the procedures The glass dissecting instruments made in the laboratory proved to be the most practical for manipulating the ganglia under the

dissecting microscope Silicone grease used in anchoring the coverslips proved to be harmless *If paraffin wax of low melting point was used for sealing the dishes it evaporated in the petri dish and formed an undesirable film over all surfaces of the petri dish although this did not interfere with fibre growth*

*General growth pattern* In the beginning of the incubation of cultures the ganglia floated freely in the culture medium However after 12 h incubation most ganglia were attached to the surface of the petri dish while others attached on it as late as after 2 days incubation Attachment as a rule proved to last until the end of the experiment

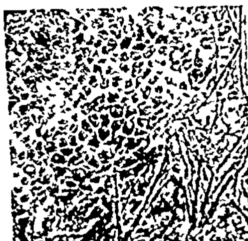
Fibre growth was first observed shortly after attachment After 12 h of incubation they formed three different types of patterns fine straight individual fibres a delicate and densely branching network of fibres, or thicker fibre trunks toward the periphery as illustrated in Fig 1 Simultaneously some connective tissue cells were seen migrating from the explant

After 24 h of incubation a distinct network of fibres was formed on the surface of the petri dish around the ganglion explant The network was often quite asymmetrical because some sectors of the fibre halo grew more rapidly than the others This asymmetry diminished during the following days when there was a phase of rapid extension of fibre network into all directions

The most outstanding features of fibre growth were its special tendency to form distinct networks between the fibres during the following 3 days (from 24 h to 4 days) and the rapid extension of the fibre network as seen in Fig 2 In this picture two ganglia were cultured so close to each other that the peripheral fibres were noticed to make contact During this time the mean daily growth of the network was over 0 2 mm the highest values for daily growth being over 0 3 mm measured on the third day of culturing

During this fibre growth period there was a simultaneous but slower migration of both supportive tissue cells and nerve cells from the explant The nerve cells migrating to the periphery also produced fibres which are clearly demonstrated at higher magnification in Fig 3 After 4 days culture period the fibre growth decreased By that time the cellular outgrowth had extended to the borders of the fibre network but seldom further The nerve cells between the fibre network tended to form groups of 3—10 cells A sign of cell migration was that the margin of the explant became indistinct and blurred Finally a monolayer of nerve cells was formed as illustrated in Fig 4

After culturing for one week the network extension slowed and during the second and third week degeneration set in if the culture medium was not changed. Degeneration appeared in the form of gradual disappearance and detachment of the fibres from the surface of the petri dish Cultures two weeks of age or older showed a gradual vacuolization of supportive tissue cells In some cultures the fibre network was in good condition even after 2 weeks of incubation without medium change and even after two months culture time some viable sympathetic neurons were recorded with fluorescence microscopy



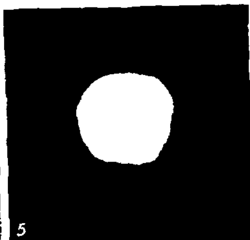




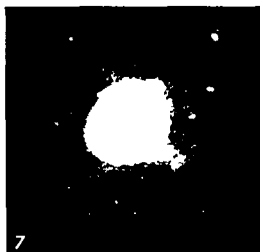
Fig 4 Sympathetic ganglion cultured for 9 days Culture medium as in Fig 1 Large group of sympathicoblasts has formed a monolayer of cells at the margin of the explant Phase contrast microscope ×300

Fig 5 Sympathetic ganglion cultured for 24 h Culture medium as in Fig 1 but without NGF There is no fibre growth from the margins of the explant Dark field microscope ×60

Fig 6 Sympathetic ganglion cultured for 4 days without NGF The explant is now degenerating and has flattened The fibroblasts have not suffered and are seen around the explant Dark field microscope ×60

In cultures incubated *without NGF* a normal attachment of the explant was observed but there was no fibre growth the explant being smooth as is seen in Fig 5 This ganglion was cultured for 24 hours without NGF After 4 days of incubation a rapid degeneration and destruction of the explant was noticed (Fig 6)

*The effect of different buffers* Comparison of TRICINE and HEPES in regard to their buffering capacities and effects on fibre growth showed no differences Using



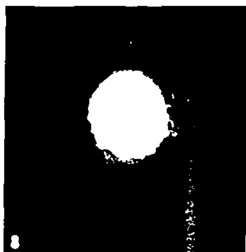




Fig. 7. Sympathetic ganglion cultured under a coverslip for 48 h. Extensive and dense fibre growth is observed around the explant. Dark field microscope. ×60.

Fig. 8. Sympathetic ganglion cultured for 48 h without a coverslip in otherwise identical conditions as in Fig. 7. The fibre growth is weaker than in Fig. 7. Dark field microscope. ×60.

Fig. 9. Sympathetic ganglion cultured under a coverslip for 4 days. The fibre growth shows two layers, the upper layer which is in focus is growing on the glass surface. Dark field microscope. ×60.

either of the buffers the pH values of the culture media after one week's incubation were stable and did not differ from the original pH recorded at the beginning of the experiment.

When pH levels in TRICINE buffer series were compared it was observed that when pH was below 6.9 fibre growth was quite satisfactory for 2 days but was followed by very rapid degeneration of the fibres and the whole explant. Test series with pH 7.0, 7.1, 7.2, 7.3 and 7.4 showed that the values between 7.1–7.4 were the

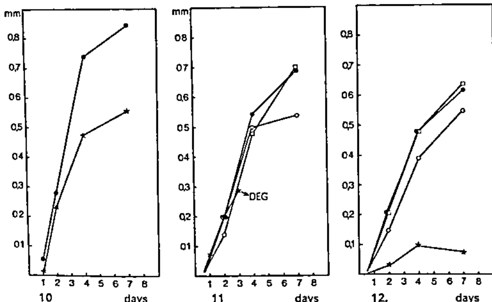



Fig 10 Effect of coverslip on fibre growth The mean halo width is measured in mm and each point represents a mean of the fibre growth of 15 sympathetic ganglia Culture medium Parker's Medium 199 buffered with TRICINE to pH 7 3 10 % calf serum, 3 0 g/l glucose 1 IU/ml NGF and 100 IU/ml G penicillin * = without coverslip ● = with coverslip

Fig 11 Effect of different glucose concentrations on fibre growth The mean halo width is measured in mm and each point represents a mean of the fibre growth of 30 sympathetic gangl a Culture medium Parkers Medium 199 buffered with TRICINE to pH 7 3 10 % calf serum 1 IU/ml NGF and 100 IU/ml G penicillin ● = 6 0 g/l concentration of glucose □ = 3 0 g/l concentration of glucose O = 1 5 g/l concentration of glucose * = 0 85 g/l concentration of glucose

Fig 12 Effect of serum concentrations on fibre growth The mean halo width was measured in mm and each point represents a mean of the fibre growth of 30 sympathetic ganglia Culture medium Parkers Medium 199 buffered with TRICINE to pH 7 3 glucose 3 0 g/l 1 IU/ml NGF and 100 IU/ml G penicillin ● = 20 % serum □ = 10 % serum O = 5 % serum * = 0 % serum

most favourable for growth Between pH 7 1—7 4 the fibre growth did not show significant differences after 7 days culturing In pH series over 7 4 up to 8 0 the fibre growth and migration of the neuronal cells suffered On the basis of these pH studies the pH value of TRICINE was adjusted to 7 3—7 4 in all further studies

Without carbon dioxide gassing Parkers Medium 199 buffered with only bi carbonate system did not remain stable Its pH level tended to rise to 8 0 before sealing of the petri dishes

*The effect of glass co erslip* The growth curve of the nerve fibres under the glass coverslip and that of the control are shown in Fig 10 The fibre growth of the explants in the micro-milieu under the coverslip was about trebled after 24 h of culture The explants maintained their better fibre growth until the third culture day After that the difference in the mean halo width remained stable After 2 days culturing the difference between the means was significant at the level $P = 0.005$ and after 4 and 7 days at the level $P < 0.0005$ as tested by the t test The favourable

influence of the coverslip on fibre growth in the beginning of the culture period is also shown in 48 h cultures with (Fig 7) and without (Fig 8) cover glass In the culture shown in Fig 9 two eparate networks were formed one grew on the lower surface of the coverslip the other on the bottom of the petri dish Between these layers there were no connecting fibres through the liquid

*The effect of glucose concentration* Fig 11 shows the growth curves obtained after culturing with different concentrations of glucose 0 85 g/l 1 5 g/l 3 0 g/l and 6 0 g/l The original glucose concentration of the culture medium was 0 85 g/l without addition of extra glucose It was noticed that fibre growth was equal to that obtained with the higher concentrations until the third day of incubation when cultures with 0 85 g/l of glucose degenerated After 7 days the cultures with higher glucose concentrations 3 0 g/l and 6 0 g/l were in better condition and showed clearly better fibre growth than those with 1 5 g/l of glucose

When the cultures with 3 0 g/l of glucose were compared with those with 1 5 g/l at the ages of 2 4 and 7 days the differences between of mean halo widths were significant P = 0 01 non significant P = 0 20 and highly significant P = 0 0005 respectively Differences of mean halo widths when the cultures of 3 0 g/l and 6 0 g/l of glucose were compared at the same ages were significant P = 0 0025 non significant P = 0 25 and non significant P = 0 45 respectively

*The effect of serum concentrations* Serum concentrations of 5 % 10 % and 20 % were tested at the same time using a control series without addition of serum

In the cultures without serum 60 % of the ganglia were totally degenerated after 2 days of incubation After 7 days of incubation only 30 % survived The surviving ganglia showed a clearly poorer fibre growth as compared with that of the cultures with serum

A serum concentration of 10 % yielded the best fibre growth No marked difference in fibre growth extension was demonstrable between these cultures and those grown in 20 % serum concentrations (Fig 12) A marked difference however existed between the density of the fibre networks The cultures in 10 % serum yielded a much denser network of fibres than those grown in 20 % serum This difference showed clearly after 4 days of culture and further increased with time Cultures grown in serum concentrations of 5 % failed to show as extensive fibre growth However a dense network was yet formed When the differences in the mean halo widths were calculated and the values with 10 % serum concentrations compared to the values with 5 % at the ages of 2 4 and 7 days the differences were significant P = 0 005 highly significant P = 0 0005 and non significant P = 0 01 When values of the 10 % cultures were compared with the 20 % cultures the corresponding results were non significant at all the above mentioned ages

## Discussion

The use of disposable equipment proved to eliminate toxic and infectious contamination of the cultures In this way the possible toxic effects of the glassware and detergents used in their washing were excluded Although the media were mostly

prepared in the laboratory infections by micro-organisms were seldom seen which proved that the Millipore filtering system was dependable After air tight closure of the petri dishes with paraffin they could be checked daily without any aseptic pre cautions

The pattern of fibre growth and its tendency to form dense networks differed markedly from the growth pattern of fibres cultured in plasma clots (Partlow and Larrabee 1971 Larrabee 1970 Dunn 1971) On the other hand Burdman (1968) described a growth pattern of anastomosing networks when dissociated sympathetic nerve cells were cultured free in liquid medium Lever and Presley (1971) and Eränkö *et al* (1972) who used culture on collagen covered coverslips in Rose chambers also reported the formation of reticulum of axonal processes in their cultured sympathetic ganglia In our cultures the fibres tended to fasciculate in the older cultures as was also pointed out by Nakai (1960) Lever and Presley (1971) and Eränkö *et al* (1972) Typical of the explant itself was its tendency to flatten on the dish to form a monolayer in the course of 2—3 weeks The e differences in growth patterns between our system and the plasma clot culture system are probably due to the lack of external support to the ganglia

Dunn (1971) described a definite contact inhibition in nerve fibres of spinal ganglia cultured in plasma clots This inhibition phenomenon was noticed also when spinal and sympathetic ganglia were cultured in the same clot No such in hibition zones were observed in our cultures between two sympathetic ganglia On the contrary when two explants were cultured close to each other there was often a more distinct network between them than in other directions This might be ex plained by the different nature of neurons in the sympathetic ganglia

If the fibres grow straight radially from the explant the width of the fibre halo directly reflects the actual length of the fibres In these cultures the circular component of the growth approaches zero In our cultures the fibres formed an anastomosing network so that there existed a distinct circular component of growth which is not reflected in the mean halo width values The density of the network formed was quite constant and so was the relation of the radial to the circular component of growth A sparse network formed in very few cultures in which the mean halo width also was smaller than in others On the ba is of these observations we consider that the mean halo width well reflects the relative fibre length These values are dependable when compared as relative values but do not reflect the actual mean fibre length which is considerably greater

HEPES and TRICINE buffers proved in our experiments efficient and non toxic to the sympathetic neurons as reported also to other tissues by Gardner (1969) Darzynkiewicz and Jacobson (1971) nad Halliburton and Becker (1971) By using these buffers it was possible to manage without the troublesome CO gassing system and to maintain stable pH conditions Although compared with TRICINE HEPES has a more favourable pKa value (for HEPES pKa value at 20° C 7 55 and $\Delta$ pKa/° C—0 014 for TRICINE pKa at 20° C 8 15 and $\Delta$ pKA/C° C—0 021 Good *et al* 1966) and has a stronger buffer capacity at the pH range used in tissue

culture conditions TRICINE was favoured because of its lower price and sufficient buffering capacity One of the disadvantages of the bicarbonate buffer system was the rapid rise of the pH in room air during preparation of the cultures When using TRICINE or HEPES buffers this unfavourable factor was avoided According to previous studies $CO_2$ and bicarbonate ions are required for the metabolism of the cells in cultures (*e g* Willmer 1965) These substances were however, present when TRICINE or HEPES was used because Medium 199 to which the buffers were added contained 0 35 g/l of bicarbonate

In our experiments the best growth was obtained with 10 % concentration of serum and increased concentrations did not improve growth With lower serum concentrations the fibre growth was markedly affected However in cultures incubated without serum some fibre growth was noticed although it was scanty and disappeared after few days This suggests that it may be possible to cultivate nerve cells in a medium where all constituents are fully controlled and no biological media are needed except purified NGF

The results illustrated in Fig 11 clearly indicate that in long term culture sympathetic ganglion cells need a high concentration of glucose to survive without medium change as has been known on the basis of the previous metabolic studies (see Larrabee 1970) Also the stimulatory effect of NGF on glucose metabolism of sympathetic neurons is well known (see Levi Montalcini and Angeletti 1968), which may further increase the glucose concentration required 3 g/l of glucose seems to be needed to provide a satisfactory supply of energy to the cultures when the medium is changed once a week

Although the nerve fibre growth was more extensive under a glass coverslip in a restricted micro milieu this method was not selected for further use because histochemical reactions could not be performed without removal of the coverslip Without the coverslip the cultured explant however presents an ideal object for the incubations for histochemical reactions and even all the steps for the fluorescence microscopical demonstration of catecholamines can be performed *in situ*

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# Cholinesterase Histochemistry of the Innervation of the Smooth Muscle Sphincters around the Terminal Intramural Part of the Ductus Choledochus in the Cat and the Dog

By

K. Kyosola

The classical neurohistological techniques have revealed a rich distribution of ganglionated nerve plexuses in the wall of the terminal intramural part of the ductus choledochus The adrenergic innervation of this minute region was recently studied using the specific fluorescence histochemical method (Eränkö 1967) in the cat and the dog (Kyösola 1973 Kyosola and Rechardt 1973) the present study concerns the cholinergic component of innervation Because the non-specific cholinesterase reaction product stains the smooth muscle excellently it was possible to study the micro-architecture of the smooth muscle sphincters in serial sections, and at the same time to delineate the scheme of the inbuilt intrinsic cholinergic nervous apparatus by demonstrating the specific cholinesterase in the adjacent sections The micromorphological study was substantiated by means of microdissection and three classical histological stain techniques

25 adult cats and 5 adult dogs were used for the present study For demonstration of cholinesterases the Gomori modification (1952) of the Koelle Friedenwald technique (1949) and the method of Karnovsky and Roots (1964) were followed. For morphological studies also paraffin techniques with H E- Toluidine blue and Weigert—van Gieson stainings were used

*Distribution of acetylcholinesterase (AChE)*

In the lamina propria of the duodenum including the core of the villi an irregular network of varicose nerve fibres or of small bundles of them was observed. The muscularis mucosae of the duodenum was innervated by a dense nerve net composed of richly beaded delicate nerve fibres in the dog this nerve net contained also some large nerve fascicles or nerve trunks and a few small ganglia In the submucosa of the duodenum, a rich distribution of AChE-positive ganglia of various size and shape connected with each others by large nerve fascicles or trunks was observed (Fig 1 The sphincter of Oddi and the sphincter of Boyden were richly innervated with AChE positive single varicose nerve fibres or small fascicles of them (Fig 2) Occasionally small AChE-positive ganglia and nerve trunks were seen. In the dog the pattern of the inbuilt intrinsic cholinergic innervation supply


Fig 1

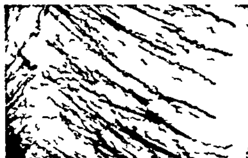

Fig 2

Fig 1 Dog Gomori AChE Ganglia and nerve fascicles of the plexus of Meissner At the left side parallel nerve fascicles penetrating the outermost layer of the sphincter of Oddi × 105

Fig 2 Dog Gomori AChE Nerve fibres and small fascicles of them course in various directions in the sphincter of Oddi surrounding the ampulla. × 105

ing the smooth sphincteric muscle was very regular exhibiting distinct regional characteristics and differences from one minute subareal to another whereas it was somewhat irregular in the cat In addition two special features were observed in the cat

1) small slender cells of an irregular form and equipped with processes
2) structures suggestive of AChE negative ganglia surrounded by AChE positive baskets of terminal ramifications of varicose nerves

In the basal 2/3 of the lamina glandularis mucosae of the ductus choledochus a nerve net with small ganglia was observed in the cat whereas this layer was devoid of AChE activity in the dog like the epithelium in both species

*Distribution of non specific cholinesterases (nsChE)*

In both species the smooth muscle and the ganglia in the submucosa of the duodenum or within the sphincters as well as the nerves in these layers and in the lamina propria of the duodenum were strongly nsChE positive In the dog the lamina glandularis mucosae of the ductus choledochus was devoid of nsChE activity whereas in the cat nsChE positive structures of several types were observed 1) ganglia composed of rounded cells 2) ganglia containing these rounded cells and in addition small cells equipped with processes 3) structures suggesting nsChE negative cells enclosed by nsChE positive baskets of varicose nerve terminals 4) long chains of slender cells with anastomosing processes The epithelium of the ductus choledochus was devoid of nsChE activity in both species

*The micromorphology of the smooth muscle sphincters*

In morphological studies the earlier descriptions of the structure of the sphincters in the choledocho-duodenal junction of the dog (Eichhorn and Boyden 1955) and

of the cat (Boyden 1957) were confirmed Correlation of the structure and function of this complex area will be discussed later in detail

## Comment

The micro architecture of the choledocho duodenal junction is utmost complex, and therefore an accurate, comprehensive and reliable description inevitably necessitates simultaneous application of several relevant techniques At the present work combining the methods of microdissection, histological stain techniques and the demonstration of nsChE in serial sections has made it possible to create a clear (3 dimensional) scheme of the organisation of the structural subunits and at the same time the demonstration of the AChE activity in adjacent sections has made possible the construction of the 3 dimensional scheme of the inbuilt intrinsic cholinergic nervous apparatus with reference to the substructures to be innervated, all this without any counterstaining Certainly combining the methods used at the present work may be useful also when studying the micromorphology and innervation of other complex regions

The structural arrangement of the sphincters allows anatomical possibilities for integrated even complex functions this necessitates an accurate nervous control In both species investigated the cholinergic innervation of the smooth muscle in the choledocho-duodenal junction was very dense in respect to the innervation of the muscularis externa of the gut an utmost complex nervous control system consisting of ganglionated nerve plexuses of 3 orders was observed the ganglia containing various cell types and presenting complex substructural features This can possibly be considered as an indicative for active complex motor (and possibly sensory) functions of the choledocho duodenal junction in contrast to the only slow gross contractions of the gut The anatomical basis for controlling the flow of bile for rhythmic suctionpressure pumping as well as for sensitive protective reflex functions seems to be provided

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From the Brain Research Laboratory E Blocket, University Hospital Lund Sweden

# Blood Flow and Oxygen Consumption of the Rat Brain in Profound Hypoxia

By

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Several recent studies have demonstrated that although a lowering of the arterial $P_O$ to 20—25 mm Hg is accompanied by a lactic acidosis in the brain there are no detectable changes in the tissue concentrations of ATP ADP or AMP (Siesjo and Nilsson 1971, Duffy *et al* 1972 MacMillan and Siesjo 1972 Lewis *et al* 1973) There are two possible homeostatic mechanisms that may explain the maintenance of a normal energy state (1) a reduction in the cerebral metabolic rate secondary to a reduced energy demand and (2) an increase in cerebral blood flow (CBF) which compensates for the decrease in arterial oxygen content Recently Duffy *et al* (1972) reported results which indicated a significant reduction in metabolic rate in mice breathing 5 % $O_2$ At a comparable degree of hypoxia we obtained results that were compatible either with an increase in CBF to about 400 % of normal or with a lesser increase in flow coupled with a corresponding reduction in metabolic rate (Lewis *et al* 1973) In the present communication a preliminary account is given of experiments designed to measure CBF and cerebral metabolic rate for oxygen ($CMR_O$ ) in profound hypoxia

Male Wistar S P F rats (380—420 g) were maintained immobilized and artificially ventilated on 70 % N O and 30 % oxygen with control of blood pressure hemoglobin concentration $P_{O_2}$ $P_{CO_2}$ and pH Body tempertaure was kept at 37° C Hypoxia was induced for 30 min by reducing the oxygen content in the inhaled gas mixture keeping the $N_2O$ concentration constant The CBF was measured with a $^{133}$xenon modification of the Kety and Schmidt technique using repeated sampling of arterial blood and of venous blood from the superior sagittal sinus during the desaturation phase (Eklof *et al* 1973) Blood was taken from the same sources for measurements of total oxygen content and $CMR_O$ was calculated as the product of the CBF and the arteriovenous difference in oxygen content ($AVD_O$ ) Control animals were obtained by maintaining animals at normal $Pa_O$ for a comparable period

The table shows that the hypoxia reduced $Pa_{O_2}$ from $>$ 100 mm Hg to about 24 mm Hg (range 22—27) and the total oxygen content in arterial blood from about 22 to a mean of 3 8 ml/100 g×min The CBF increased to almost 500 ml/100 g× min *i e* to more than 400 % of normal but the $CMR_{O_2}$ remained constant

TABLE I Influence of hypoxia on blood flow (CBF) and on oxygen consumption ($CMR_O$) of rat brain The arteriovenous difference in oxygen content ($AVD_O$) was measured between the femoral artery and the superior sagittal sinus Means ± S E

| | $Pa_O$ mm Hg | $[O_2]_a$ ml/100 ml | [O ] ml/100 ml | $AVD_{O_2}$ ml/100 ml | CBF ml/100 g × min | $CMR_O$ ml/100 g × min |
|---|---|---|---|---|---|---|
| Normal | 140 | 22 28 | 12 92 | 9 26 | 114 | 10 3 |
| n = 6 | ±6 | ±0 7 | ±0 7 | ±0 7 | ±6 | ±0 3 |
| Hypoxia | 24 2 | 3 82 | 1 71 | 2 11 | 489 | 10 0 |
| n = 6 | ±0 9 | ±0 2 | ±0 1 | ±0 2 | ±45 | ±0 7 |

The present results demonstrate that the maintenance of normal tissue concentrations of ATP ADP and AMP in profound hypoxia is not due to a decrease in the rate of cerebral oxygen consumption Thus provided the closed box method used by Duffy *et al* (1972) gives a valid measure of metabolic rate in hypoxia it must be concluded that accidental hypothermia occurring in their animals was responsible for the calculated decrease in metabolic rate It follows from the present experiments that the increase in cerebral blood flow is responsible for the homeostatic regulation of the energy state of the brain in hypoxia Further studies of the mechanisms that elicit the increase in CBF therefore seem warranted

## Acknowledgements

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Acta physiol scand 1974 90 283—285
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# Possible 'Dynamic' Component in the Myogenic Vascular Response Related to Pulse Pressure Distension

By

STEFAN MELLANDER and STEFAN ARVIDSSON

Vascular tone in sections with the single unit type of smooth muscle as in precapillary resistance vessels can at least partly be conceived of as the integrated result of phasic smooth muscle twitches or tetani initiated by action potentials conducted from pacemaker cells (for ref see Mellander and Johansson 1968 Johansson 1971) Stretch of the vascular smooth muscle evokes a contractile response apparently due to increased pacemaker activity and more frequent bursts of action potentials (Johansson and Bohr 1966) which can explain the myogenic constrictor response of the resistance vessels to raised transmural (blood) pressure (myogenic component in the autoregulation of blood flow Folkow 1964) Myogenic vascular reactivity and autoregulation in vivo has in the past been analysed in response to changes of mean arterial pressure (below called static stretch stimulus) It seems possible however that the vascular smooth muscle mechanoreceptor effector via its electrical events is sensitive also to a dynamic stretch stimulus created by repetitive pulse pressure distension or by the rate of pressure change The present in vivo experiments lend support to this hypothesis

The study was performed on the sympathectomized vascular bed of the lower leg muscles in the cat where the popliteal artery and vein formed the sole vascular connections with the animal A shunt circuit, connected via a T tube to an artificial airfilled Windkessel system was inserted between the femoral and popliteal artery Arterial pulsatile and mean pressures were monitored from the shunt circuit distal to the Windkessel system and pressure in the latter was adjusted to the prevailing mean arterial pressure Popliteal vein pressure and venous outflow of blood from the muscle region were also continuously recorded Regional arterial and venous pressures could be adjusted to desired levels by a micrometer screw clamp around the arterial shunt and by changing the height of the venous outflow tubing When the connection to the Windkessel system was closed the vascular bed was exposed to normal pulse pressure oscillations averaging [illegible]4 ± 3 8 mm Hg and these oscillations although somewhat reduced were clearly transmitted to the arterial micro vessels as evidenced by recordings of small artery pressure The pulse pressure oscillations could be damped out instantaneously to an average value of 8 ± 1 2 mm Hg by opening the connection to the Windkessel system Vascular resistance was compared during pulsatile and non pulsatile conditions in different tests both when myogenic tone was maintained (below called normal tone) and when it was abolished by close arterial infusion of papaverine (passive vascular bed Data are given as mean values ± S E

*I Effects of pulse pressure oscillations per se on vascular resistance* Vascular resistance was analysed during non pulsatile and pulsatile conditions in paired experiments with identical mean perfusion gradient. In experiments with normal

vascular tone a sudden shift from non pulsatile to pulsatile pressure led to a gradual increase of vascular resistance usually reaching a new steady state within 30—60 s the time characteristics indicating that the effect was caused by an active smooth muscle response Steady state vascular resistance during pulsatile conditions sometimes exceeded that during non pulsatile conditions by more than 20 % but the effect was usually less pronounced, the average resistance increase for 129 such paired observations being $6 \pm 0.5$ % ($p < 0.001$) In the passive vascular bed, on the other hand the shift from non pulsatile to pulsatile pressure led to an immediate and maintained decrease of vascular resistance by an average value of $10 \pm 1.4$ % (32 paired observations) This decrease of resistance (increase of blood flow) is most likely explained by the non linear characteristics of the pressure flow curve for the passive vascular bed, the curve being convex towards the pressure axis passive distension and passive resistance decrease during the systolic pressure peak causes a non proportionately big increase of flow which more than outbalances the flow decrease during the diastolic phase The qualitatively different response in the normal and passive vascular bed suggests that the pulse pressure induced stretch of the vascular smooth muscle initiates a myogenic constrictor response which overrules the effect of passive distension The dynamic stretch stimulus may however not necessarily be responsible for an average myogenic resistance increase of precisely 16 % (difference between the effect in the normal and passive vascular bed) due to somewhat different vessel wall distensibility characteristics arterial input impedance etc in the two situations

2 *Myogenic response to increased mean distending pressure under non pulsatile and pulsatile conditions* A critical experiment for the demonstration of myogenic autoregulatory responses is to study the blood flow or resistance variations during altered vascular distending pressures produced by simultaneous and equivalent changes in mean arterial ($P_A$) and venous ($P_V$) pressure (mean pressure head kept constant) If pulse pressure is a trigger mechanism for myogenic automaticity a moore pronounced myogenic constrictor response would be expected to a given rise of mean distending pressure $(P_A + P_V)/2$ during pulsatile than non pulsatile conditions The effects on muscle vascular resistance to standardized identical increases of $(P_A + P_V)/2$ were studied in paired experiments with non pulsatile and pulsatile pressure during normal vascular tone (22 paired observations) and in the passive vascular bed (10 paired observations) The induced increase of $(P_A - P_V)/2$ averaged $22 \pm 1.0$ mm Hg In the normal vascular bed the change from low to high mean transmural pressure led to an increase of regional resistance by $13.5 \pm 1.4$ % during pulsatile and by $7.2 \pm 1.4$ % during non pulsatile conditions the difference being 6.3 % which is significant ($p < 0.01$) In the passive vascular bed the rise of transmural pressure instead decreased resistance by an average value of $20 \pm 4.6$ % The distending effect of increased transmural pressure during constant mean pressure head seen in the passive vascular bed thus seemed overruled by a myogenic constrictor response in the normal vascular bed but more effectively so during pulsatile than non pulsatile conditions

3 *Autoregulation of blood flow during pulsatile and non pulsatile pressure conditions* If besides a static stimulus there is a dynamic stimulus for the myogenic response myogenic autoregulation of blood flow might be more efficient under pulsatile than non pulsatile conditions Experimental tests suggested this to be the case Blood flow at any given perfusion pressure in the range from about 80 to 140 mm Hg was found significantly larger during non pulsatile than pulsatile conditions

*Comments* The present data taken together indicate that there is a static as well as a dynamic possibly rate sensitive component in the myogenic response to stretch of the vascular smooth muscle implying constrictions of the resistance vessels in response to increases of mean as well as pulse pressure and dilator effects in response to decreases of either of these two pressures The cellular mechanism behind the dynamic vascular smooth muscle response to stretch remains to be shown but may very well be the result of increased electrical activity in the pacemaker cells Thus studies on a myogenically active vascular smooth muscle preparation with regularly timed spontaneous bursts of action potentials and contractile responses showed that a given stretch could initiate a burst of action potentials and a contractile response in the quiescent period if the stimulus was applied rapidly but not if it was applied more gradually (Johansson and Mellander to be publ ) — A dynamic myogenic vascular response may be of great functional significance in the sense that pulse pressure could be a factor involved in the establishment and the modulation of basal vascular tone in normal and pathophysiological situations If so the Windkessel function of aorta and the large arteries might be indirectly involved in the control of the peripheral circulation via its effect on pulse pressure A changed elasticity modulus of the Windkessel vessels caused by adrenergic smooth muscle activation or by arteriosclerotic stiffening might lead to reinforcement of basal vascular tone in the periphery and in the latter case indirectly be one causal factor in the development of hypertension

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Acta physiol scand 1974 90 286—288

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# Stereoselectivity of Presynaptic α-Adrenoceptors Involved in Feedback Control of Sympathetic Neurotransmitter Secretion

By

LENNART STJÄRNE

The probability for secretion of noradrenaline (NA) from sympathetic nerve terminals as a result of depolarization by the propagated nerve impulse seems to be restricted by an α adrenoceptor mediated feedback control mechanism (Häggendal 1970) The specific adrenoceptors involved in this control appear to be located presynaptically on the nerve terminals themselves (Farnebo and Hamberger 1970 Langer 1970) This implies that the sympathetic nerve terminals are capable of sensing changes in the NA concentration in their immediate vicinity and of adjusting their secretory activity accordingly (Starke 1972 Stjärne 1973 a)

The present paper reports the finding that the α adrenoceptors involved in feedback control of NA secretion from the sympathetic nerves of guinea pig vas deferens are distinctly stereoselective at concentrations where exogenous (−) NA reduced the secretion of labelled transmitter secretion to one half (+) NA was completely without effect

The experiments were carried out in isolated vasa deferentia from 10 guinea pigs weighing 300—350 g After preincubation of the tissue at 30° C for 30 min in 1 ml Tyrode solution containing 10 μCi (260 ng) of ³H l NA (New England Nuclear Corp) the washed preparation was mounted in a bath for superfusion with Tyrode solution at 2.5 ml/min and 30° C ³H NA secretion was evoked by supramaximal field stimulation trains of 300 pulses 1.5 ms in duration and at a frequency of 1 Hz were applied with about 16 min intervals Since practically all of the radioactivity retained in the washed preparation was intact ³H NA since desipramine $6 \times 10^{-7}$ M and normetanephrine 10 M were added to prevent rebinding of NA and since the evoked rise in efflux of ³H was completely blocked by tetrodotoxin $5 \times 10$ g/ml it is assumed that the evoked fractional rise in efflux of total ³H

$$\frac{\text{evoked rise in efflux of } ^3\text{H}}{\text{total } ^3\text{H in tissue at the time of stimulation}}$$

gives a valid measure of the neural secretion of ³H NA

The results are shown in Fig 1 which represents 3 of the experiments in the series the fractional secretion of ³H NA per stimulus (Δt) was somewhat high

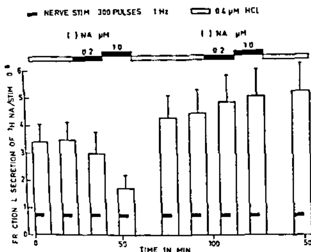



Fig 1 Fractional secretion of ³H NA per stimulus (Δt) from isolated superfused field stimulated guinea pig vas deferens Effects of (−) NA and of (+) NA 3 expts Vertical bars SE

when compared to other series and also showed an unusual and marked tendency to increase with time (*cf* Stjarne 1973 a) In accordance with previous findings (Stjarne 1973 a b) exogenous (−) NA dose dependently and reversibly depressed Δt (at 1 µM to 46.8 ± 4.4 % of Δt during the preceding control stimulation $p < 0.001$) However (+) NA at the same concentrations was completely without effect The same results were obtained in other experiments where the two enantiomers of NA were administered in the reverse order Since a 10 fold rise in desipramine did not alter the depressing effect of exogenous (−) NA on Δt it seems highly unlikely that the observed inhibitory effect of (−) NA on secretion of labelled transmitter could represent an artefact and be due to uptake and subsequent preferential secretion of the unlabelled exogenous NA The complete lack of inhibitory effect of (+) NA also argues against this possibility since the affinity of this enantiomer of NA for uptake into the adrenergic neurons of vas deferens at least in rabbit (Hendley and Snyder 1972) is almost one half of that of (−) NA. Furthermore (−) adrenaline which has considerably lower affinity for uptake into the adrenergic neurons of guinea pig vas deferens than (−) NA (Jarrott 1970) was found to be even more potent than (−) Na as inhibitor of the secretion of labelled transmitter in guinea pig vas deferens (Stjarne to be published)

Thus in conclusion The specific probably presynaptic α adrenoceptors involved in the feedback control of sympathetic neurotransmitter secretion in isolated guinea pig vas deferens show a marked stereoselectivity in favour of the naturally occurring (−) enantiomer of NA

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# Aspects of the Relative Roles of Peripheral Vasoconstriction and Vagal Bradycardia in the Establishment of the "Diving Reflex" in Ducks

By

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## Abstract

BLIX A S E L GAUTVIK and H REFSUM *Aspects of the relative roles of peripheral vasoconstriction and vagal bradycardia in the establishment of the diving reflex in ducks* Acta physiol scand 1974 *90* 289—296

The effects of $\alpha$ adrenergic (Phenoxybenzamine) and parasympathetic (Atropin) blockade on the cardiovascular response to diving in the duck has been studied With no significant reduction in the immediate slight fall in heart rate $\alpha$ adrenergic blockade caused peripheral vasodilatation and virtually abolished the more delayed phase of profound bradycardia during diving Atropinization eliminated both elements of the diving bradycardia while the arterial and venous pressure greatly increased suggesting that intense activation of the vasoconstrictor fibres was maintained during submersion It is concluded that the gradual but profound intensification of the initial slight bradycardia in the diving duck is a matter of a secondary reflex adjustment to the pressure level


The conspicuous diving bradycardia has been much studied (for a review see Andersen 1966) while comparatively little attention has been paid to the vaso constriction simultaneously developed (*e g* Irving 1938 Bron *et al* 1966) There is little doubt however that an intense systemic vasoconstriction together with a consequent central displacement of the blood volume constitutes an absolute pre requisite for oxygen preservation in ducks and other expert divers This is clear from the profound reduction and redistribution of cardiac output seen during submersion in the duck (*e g* Folkow Nilsson and Yonce 1967 Folkow 1968) Thus the brady cardia taking place during a dive may to some extent be a secondary compensatory adjustment to the vasocon triction The cardiovascular respon es to diving has there fore been examined in ducks after elimination of the vasoconstrictor fibre influence by means of high doses of the potent $\alpha$ adrenergic blocking agent phenoxy benzamine However since this drug is known to exhibit some anticholinergic ef fects both *in vitro* and *in vivo* tests were performed to explore the efficiency of this

side effect on the duck heart and its vagal control. Furthermore, in order to throw additional light upon the cardiovascular adjustments to diving, some studies were also performed using atropinized animals.

## Material and methods

Twenty-eight domestic ducks of both sexes weighing about 3 kg were used in this study.

*Diving experiments.* The animals were restrained ventral side down, and diving was simulated according to Andersen (1959). The brachial artery and vein were cannulated with polyethylene catheters (PP 100) during local anesthesia, and central arterial and venous pressure were recorded using Statham transducers (P23H & P23BB) with a Beckman–RS polygraph.

After the normal cardiovascular responses to diving had been recorded (Fig. 1), the α-adrenergic blocking drug phenoxybenzamine* was infused intravenously in a total amount of 10 mg/kg b.wt. This dose was diluted in saline to a volume of 10 ml and administered intermittently, 1 ml each 10 min period. To obtain the maximal drug effect, the recordings were made during the second 1 h period after the last infusion (Goodman and Gilman 19[illegible]).

Similar diving experiments were performed during parasympathetic blockade induced by intravenous administration of atropine (1 mg/kg).

*Experiments concerning cardiac effects of phenoxybenzamine.* To test the myocardial effects of phenoxybenzamine *in vitro*, the ducks were killed by a blow to the head, the heart was excised immediately, and the atria quickly removed and suspended in a water jacketed organ bath containing oxygenated modified Ringer solution at 32 °C. This solution had the following composition (all ions in meq/l): $Na^+$ 143.4, $K^+$ 5.3, $Ca^{++}$ 5.1, $Mg^{++}$ 2.3, $Cl^-$ 126.4, $H_2PO_4^-$ 2.4, $HCO_3^-$ 25.0, $SO_4^{--}$ 2.3 and Glucose 1.8 mg/ml. The solution was continuously aerated with 95 % $O_2$ and 5 % $CO_2$, the pH was 7.4.

A preload of 2 g was added to the preparation and isometric contractions were recorded by a Grass force displacement transducer (FTO 3C) connected to a Grass polygraph. After a 30 min period of equilibration the atria were electrically stimulated, using square wave pulses of 0.5 ms duration with bipolar platinum electrodes. In order to measure the threshold values required to drive the atria, the stimulus frequency was first set 10 % higher than the spontaneous rate. The intensity was increased until the atria followed the rate of the extrinsic stimulation. Phenoxybenzamine was then added to the organ bath giving final concentrations ranging from $5 \times 10^{-6}$ M to $5 \times 10^{-4}$ M. Eight min later the recordings were repeated. The preparations were washed twice with Ringer solution and the procedure was repeated four times after equilibration periods lasting 10 min.

The effect of phenoxybenzamine on the negative inotropic effect of acetylcholine (ACh) was examined in separate experiments in which strips of left atrias were electrically stimulated with 100 impulses/min. The absolute changes in contraction amplitude were measured over a range of ACh doses from $5 \times 10^{-8}$ M to $5 \times 10^{-4}$ M. In order to determine the influence of phenoxybenzamine on the negative inotropic effect of ACh, the α-adrenergic blocker was added to the organ bath to a final concentration of $2.5 \times 10^{-6}$ M. Then the ACh administrations were repeated. The bath was washed twice between the tests and control values were recorded immediately before each ACh administration. Each series of tests was repeated four times.

Mean log dose response curves for ACh were constructed in the absence and the presence of phenoxybenzamine $2.5 \times 10^{-6}$ M. The significance of these *in vitro* anticholinergic effects of phenoxybenzamine for the situation *in vivo* was examined as follows: the brachial artery and vein were cannulated with PP 100 catheters during local anesthesia, arterial blood pressure being measured by means of a Statham P 23 Dc pressure transducer connected to a Beckman-RS oscillograph. The ducks were then anesthetized with chloralose (40 mg/kg) and urethane (750 mg/kg) and placed in supine position during artificial respiration at a rate of 30 per min and a tidal volume of 35 ml.

The vagal nerves were isolated in the neck and sectioned under cold blockade induced by exposure to ice. The peripheral end of the left vagal nerve was placed on a platinum electrode connected to a Biotronic stimulator and stimulated at 6 imp/sec with square wave pulses of 1 ms duration. During supramaximal stimulation of the left vagal nerve at this frequency a heart rate was induced which corresponded to that seen in the intact "resting" duck. Such direct vagal stimulations were performed both before and after i.v. phenoxybenzamine ad-

* Phenoxybenzamine hydrochloride (Dibenzyline) Smith, Cline and French Labs. Ltd. Herts., England.

ministration with recordings of the changes in heart rate induced by the direct vagal stimulations Finally atropine was given and the vagal stimulations were repeated

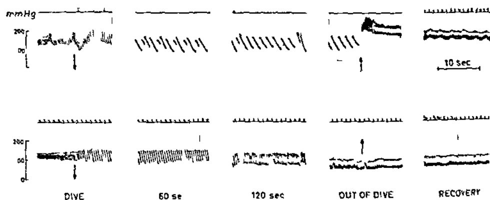
## Results

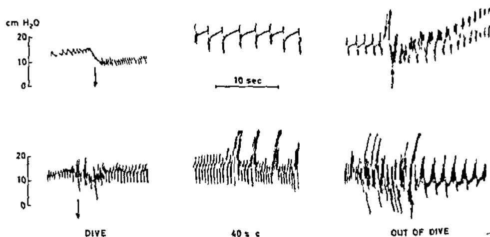
Normally the domestic ducks used in these experiments would easily tolerate submersion for 8 min In control dives of 3 min duration which were used as a standard the heart rate (HR) fell from 213 ± 22 beats per minute (BPM) to 47 ± 11 BPM The heart rate was 250 ± 49 BPM in the recovery phase 5 s after emersion Central arterial blood pressure (CABP) remained almost constant during diving being 131/72 in the predive period and after 60 s of submersion 127/65 mm Hg

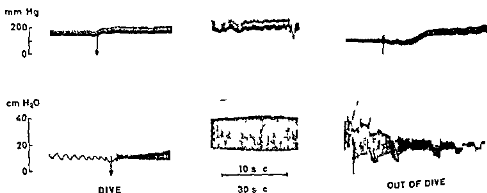
It was verified in separate experiments that the response of the animals to phenoxybenzamine was dependent upon the dose administered In the rest of the experiments 10 mg/kg b wt was therefor used as this seemed to be the smallest dose ensuring a virtually complete vasoconstrictor fibre blockade No obvious side effect were observed with this dose whereas doses of 20 mg/kg b wt killed the animals

When diving was repeated after phenoxybenzamine a fall in heart rate from 216 ± 60 to only 122 ± 18 BPM was observed while CABP fell from 145/92 mm Hg to 12?/58 60 s after the dive was started In the recovery phase HR increased to an average of 353 ± 23 BPM but in spite of this the usual rise in blood pressure was largely abolished Fig 1 illustrates a typical experiment with the effect of submersion on heart rate arterial pressure (A) and central venous pressure (B) before and after administration of phenoxybenzamine Note that an immediate but unconspicuous reduction in heart rate occurs in both cases while the more delayed but profound bradycardia with concomitantly increased venous pressure only appears in the intact animal In the phenoxybenzamine treated duck on the other hand a progressive fall in arterial pressure was recorded Fig 1 C illustrates the changes in heart rate arterial and venous pressures after atropinization where the submersion bradycardia is absent in spite of a considerable rise in arterial and central venous pressure

The *in vitro* experiments on isolated duck atria were performed to explore the possibility of a direct influence of phenoxybenzamine on the myocardial cells and/or their vagal control elements After the atria had been placed in the organ bath the force of the rythmic contraction gradually increased until a steady state was reached after about 20 min Addition of phenoxybenzamine in doses ranging from $5 \times 10^{-6}$ M to $5 \times 10^{-4}$ M hardly caused any relevant alterations of rate or contractile force and the work index (the product of atrial rate/min and the contractile peak tension in mg Loeb 1965) remained approximately constant (Fig 2 The electric intensity required to stimulate the atria at a frequency 10 % higher than the spontaneous rate in the control period was neither significantly influenced by the phenoxybenzamine administration (Table I) However phenoxybenzamine ($2.5 \times 10^{-6}$ M) induced a parallel shift to the right of the log dose response curve for acetylcholine concerning its negative inotropic effect on the atria In other words under these *in vitro* conditions phenoxybenzamine exerted a slight atropinelike action in the dose range mentioned (Fig 3)


A
mmHg
200
100
10 sec
200
100
0
DIVE
60 sec
120 sec
OUT OF DIVE
RECOVERY


B
cm $H_2O$
20
10
0
10 sec
20
10
0
DIVE
40 sec
OUT OF DIVE


C
mm Hg
200
0
cm $H_2O$
40
20
0
10 sec
DIVE
30 sec
OUT OF DIVE

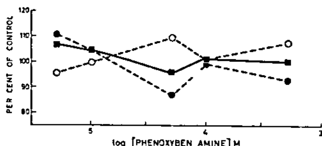



Fig 2 Log dose response curves showing the inotropic (O—O) and chronotropic (●—●) effects of phenoxybenzamine and upon work index (■—■) of the isolated duck atria. Each value represents mean of four experiments and is calculated in per cent of the control values

To test whether the phenoxybenzamine dosage used *in vivo* to block the vasoconstrictor fibres (10 mg/kg) exerted any atropinelike effect on the vagal control of heart rate direct graded stimulations of the left vagal nerve were performed in vagotomized anesthetized ducks before and after administration of phenoxybenzamine (10 mg/kg) Fig 4 illustrates that this dosage does not significantly reduce the vagal influence on the heart

During supramaximal stimulation at 6 Hz of the left vagal nerve a heart rate comparable to that of the normal resting duck was obtained whether or not α adrenergic blockade had been performed This implies that the greatly reduced bradycardia during diving cannot be ascribed to any direct interference by phenoxybenzamine on the vagal control of the heart rate

## Discussion

The α adrenergic blocking drug phenoxybenzamine has been shown to exert a profound effect on the diving reflex in ducks Not only does it interefere with the peripheral vasoconstriction it reduce the vagal bradycardia markedly as well

TABLE I Effects of phenoxybenzamine on contractile force work index and the electrical th eshold on the isolated duck at ia *in vit o*
Each value (mean ± S E ) p ents the mean of 4 expts and s calculat d in per cent of the values obtained n the control p riod

| Concentration (Molar) | Atrial contractile force | Atrial frequency | Work index | Electrical threshold |
|---|---|---|---|---|
| $5 \times 10^{-6}$ | 95.6 ± 4.7 | 110.9 ± 6.9 | 106.8 ± [illegible]8 | 98.1 ± 1.1 |
| $10^{-5}$ | 100.0 ± 3.9 | 104.9 ± 1.8 | 104.8 ± 3.9 | 100.5 ± [illegible]2 |
| $5 \times 10^{-5}$ | 110.3 ± 3.7 | 87.1 ± 6.4 | 96.[illegible] ± 4.4 | 105.8 ± 3.9 |
| $10^{-4}$ | 101.0 ± 0.7 | 99.7 ± 0.3 | 101.0 ± 0.9 | 97.1 ± 3.9 |
| $5 \times 10^{-4}$ | 108.0 ± 1.6 | 93.1 ± 2.4 | 100 ± [illegible]4 | 10[illegible].8 [illegible]0 |

Fig 1 Typical tracings of A) ntral arterial pressu e and B) central venous pressure re ponse to diving in normal (upper tracing) and α ad energic blocked animals (lower tracings) C) Typical changes in central arterial pressure (upper tracing) and central venous pres ure (lower tracing) upon a one minute di e afte atropinization of an animal

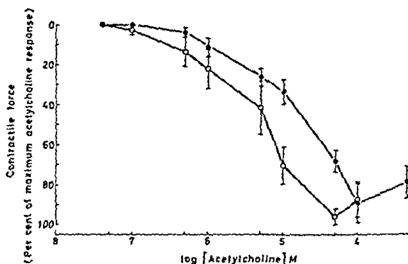



*Fig 3 Log dose response curves showing the negative inotropic effect of acetylcholine in the* presence (●—●) and absence (○—○) of phenoxybenzamine ($2.5 \times 10^{-6}$ M) Each value (mean ± S E ) *represents the mean of 4 expts*

These results raise the question in which way the α adrenergic blocking drug interferes with the heart and its vagal control *In vitro* tests concerning the phenoxybenzamine effects on isolated duck atria revealed that spontaneous rate contractile force and stimulation threshold were virtually unaffected by the drug in the concentrations used while a moderate anticholinergic effect could be demonstrated

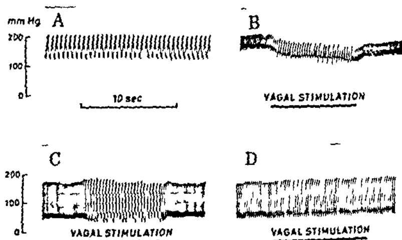



Fig 4 *Recording* of central arterial blood pressure of two representative anesthetized ducks. UNTREATED ANIMAL A before vagotomy B—after vagotomy during supramaximal left vagal stimulation at 6 Hz α ADRENERGIC BLOCKED ANIMAL C—during supramaximal left vagal stimulation at 6 Hz and D—same as C except for addition of atropine A & B and C & D obtained in 2 different experiments

when phenoxybenzamine was added to the organ bath in a concentration of 2 $5 \times 10^{-6}$ M This concentration led to a parallel shift to the right of the log dose response curve for acetylcholine However *in vivo* tests where direct graded stimulations of the vagal nerves were performed showed that the negative chronotropic vagal effect on the heart was largely unaffected when phenoxybenzamine was given in the same concentrations as those used for blocking the vasoconstrictor fibres in the diving experiments

These results indicate that the absence of the profound diving bradycardia is not primarily a pharmacological side effect of phenoxybenzamine directly on the heart and its vagal control It must be a consequence of the vasoconstrictor fibre blockade When complete such a blockade must lead to a peripheral vasodilatation during diving The presence of such a dilatation was indicated by the findings that arterial pressure gradually fell during submersion while central venous pressure did not exhibit its usual rise Furthermore the marked rise in arterial pressure during the immediate recovery period was absent although the heart rate did not differ from that seen in untreated animals In addition the diving capacity was conspicuously reduced as would be expected if the vasoconstrictor fibres fail to restrict systemic blood flow to muscles and viscera

Murdaugh *et al* (1968) have examined the interaction between bradycardia and peripheral vasoconstriction from the opposite point of view as in the present study These authors demonstrated that seals develop strong peripheral vasoconstriction during diving even when intracardiac pacing of the heart prevents the bradycardia response These results point to the vasoconstrictor fibre activation as a primary and forceful element in the diving response and not mainly to be considered as a secondary adjustment to a primary heart inhibition This does of course by no means deny that superimposed reflex influences probably are involved too modifying the vasoconstrictor response

Also the present results indicate that the neurogenic vasoconstriction is a primary and crucial element in the diving response In some animals however the very initiation of the vagal bradycardia is evidently also a primary event as indicated *e g* by the almost immediate and sometimes even anticipatory bradycardia of the seal (*e g* Scholander 1940) or the coypu (Folkow Lisander and Öberg 1971) Moreover in this study also the ducks exhibited an immediate though usually slight bradycardia upon submersion whether or not the animal had been treated with phenoxybenzamine (Fig 1) This initial slight reduction in heart rate often occurs from one beat to the other *i e* before the vasoconstrictor response could have been initiated Therefor it can hardly be ascribed to a secondary reflex adjustment of the heart to a primary vasoconstrictor effect However in the duck the profound and functionally important phase of bradycardia is somewhat more delayed in onset and this intense phase of bradycardia was virtually abolished by phenoxybenzamine

During submersion which naturally supresses respiratory movements the primary reflex response on the heart elicited from the chemoreceptors is a vagal bradycardia (*e g* Daly *et al* 1967) Since the failing peripheral vasoconstriction after phenoxy

benzamine in the present experiments must lead to a more rapid elimination of the blood oxygen stores by peripheral O consumption blood hypoxia obviously develops far more rapidly in the phenoxybenzamine treated animals than in normal ones This would if anything intensify the chemoreceptor reflex inhibition of heart rate via the vagal nerves but there was no signs of vagal bradycardia in the animals treated with phenoxybenzamine in the later stages of the dive when hypoxia would be especially intense By exclusion therefore it appears that some other reflex element normally contributes in an important way to the delayed phase of intense bradycardia in the diving duck The phenoxybenzaminetreated animals presently studied were characterized by a falling arterial pressure and an absence of the normal rise in cardiac filling pressure as combined with an absence of the intense bradycardia respon e during submersion These results indicate that arterial and/or intraventricular mechanoreceptors both known to elicit pronounced reflex brady cardia when activated (e g Oberg and Thoren 1972) may contribute to the gradual ly more intense bradycardia seen during submersion in the duck Moreover the pronounced bradycardia component rather than the vasoconstriction eems to be crucially dependent on such secondary mechanoreseptor reflexes Both the results obtained by Murdaugh et al (1968) and those presented here concerning the effects of atropinization (e g Fig 1 C) support such a conclusion

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Acta physiol scand 1974 90 297—302
From the Department of Physiology Gymnastik och idrottshogskolan Stockholm Sweden

# Time Course for Refilling of Glycogen Stores in Human Muscle Fibres Following Exercise Induced Glycogen Depletion

By

KARIN PIEHL

Received 11 April 1973

## Abstract

PIEHL, K *Time course for refilling of glycogen stores in human muscle fibres following exercise induced glycogen depletion* Acta physiol scand 1974 *90* 297—302

Muscle biopsy samples were obtained from the thigh muscle of 4 subjects before and after 2 h of work and at selected intervals during the following 46 h when a carbohydrate enriched diet was consumed Mean glycogen content declined 103 (from 125 to 22) mmol glucose units $\times kg^{-1}$ following exercise 5 and 10 h after consuming the carbohydrate enriched diet muscle glycogen increased to 64 and 86 mmol glucose units $\times kg^{-1}$ respectively During the first 5 h there was a marked storage of glycogen in the muscle which was related to the carbohydrate intake but pre exercise concentrations of muscle glycogen were observed first after 46 h The increase in glycogen occurred in both fibre types but the fast twitch fibres replenished their glycogen somewhat faster than did the slow twitch fibres suggesting a higher glycogen synthetase activity At glycogen concentrations above 80—90 mmol no differences in the glycogen content of the two fibre types could be discerned with histochemical methods

Glycogen in human skeletal muscle is broken down during exercise (Hultman 1967) This may occur selectively in one fibre type depending on work conditions (Gollnick *et al* 1973 a 1973 b) *It takes a certain time to refill the depleted muscle glycogen* stores With a carbohydrate enriched diet the glycogen level will in 2—3 days return to normal values (Bergstrom *et al* 1967 Saltin and Hermansen 1967) In order to obtain extremely high muscle glycogen levels an exercise induced glycogen depletion is of importance (Bergstrom and Hultman 1966)

Considerable information is available on the regulation of glycogen synthesis (Adolfsson 1972 Bergstrom Hultman and Roch Norlund 1972) When the glycogen content of the muscle is low the rate of glycogen synthesis is well related to the activity of the enzyme glycogen synthetase (Bergstrom *et al* 1972) In rodents this enzyme has been shown to have a greater activity in the red than in the white fibres of skeletal muscle (Hess and Pearse 1961 St George Stubbs and Blanchaer 1965 Bocek and Beatty 1966) This may suggest a differential rate of refilling for glycogen in the two different fibre types found in human skeletal muscle

The intent of this study was to follow the time course for glycogen storage in

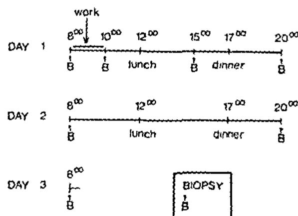



Fig 1 A schematic illustration of the protocol

individual muscle fibres after exercise induced glycogen depletion and to evaluate how this refilling of stores is correlated to carbohydrate intake Thus the caloric intake and its carbohydrate content was controlled and muscle biopsies were taken before and at given time intervals after food intake It was found that about 60 % of the total increase in muscle glycogen content took place within 10 h after the exercise induced depletion During this period most of the intake of carbohydrate was found stored in the muscle The increase in glycogen content after exercise was more enhanced in the white than in the red fibres

## Subjects and Methods

Four healthy male physical education students were used as subjects They were 25 (21—32) years old 181 (177—186) cm tall and weighed 70 kg (63—75) Their average maximal oxygen uptake was 3 95 l/min (53—58 ml/kg × min)

Muscle samples from the vastus lateralis were obtained using the biopsy technique (Bergstrom 1962) The samples were divided into two parts One part was frozen in liquid nitrogen and stored at −80 C until subsequently analyzed for total glycogen (Karlsson Diamant and Saltin 1971) A second part for histochemical analysis was also frozen in liquid nitrogen and sections were cut at −20 C as previously described (Gollnick *et al* 1972 a) Myofibrillar adenosine triphosphatase (ATPase) activity was determined (Padykula and Herman 1955) and the distribution of glycogen estimated from the periodic acid Schiff (PAS) reaction (Pearse 1961) Muscle fibres were identified as slow twitch (ST) and fast twitch (FT) on the basis of myofibrillar ATPase activity at pH 9 4 (Gollnick *et al* 1972 a) The relative glycogen distribution in the fibres was subjectively rated under a light microscope as dark moderate light and negative for the PAS staining as previously described (Gollnick *et al* 1972 b) This method was based upon that described by Kugelberg and Edstrom (1968) The relationship between PAS staining intensity and incubation time was determined by incubating sections of varying thickness 5 10 15 and 20 μm) for 2 5 10 or 20 min In no instance did section thickness or incubation time significantly effect the staining intensity and pattern

## Procedure

The subjects consumed a diet composed of 60 % carbohydrate (4000 kcal/24 h 1 kcal = 4 19 kJ) during the 46 h after depleting their muscle glycogen Lunch (1200 kcal) and dinner (1800 kcal) were prepared for the subjects in the laboratory whereas breakfast and snacks (300—500 kcal) were taken at home Their diet prior to the experiment was not controlled

The subjects reported to the laboratory at 8 a m and proceeded to reduce the glycogen stores of their vastus lateralis muscle by exercising for 2 h (Fig 1) The first hour consisted of endurance exercise (swimming skiing running and bicycling) and the second hour consisted

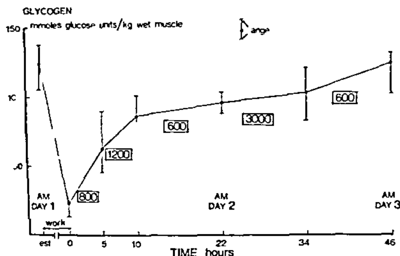



Fig 2 Total muscle glycogen concentrations during the experiment. The numbers in the squares show carbohydrate intake expressed in mmoles of glucose

of repeated periods of brief maximal exercise performed to exhaustion on a bicycle ergometer

Muscle samples were taken before and after exercise During the remainder of the experiment the subjects did not participate in any physical activity except slow walking As shown in Fig 1 3 h elapsed between meals and the muscle biopsy samples on the first day During the rest of the experiment muscle samples were taken only in the morn ng (1 h after breakfast) and in the evening (3 h after dinner)

## Results

Changes in total glycogen concentration are present in Fig 2 Pre-exercise values averaged 125 (112—131) mmol glucose units×kg [1] After 2 h of exercise this had declined to 23 (13—38) mmol glucose units×kg [1] This was a marked decrease and of the magnitude that could be anticipated after 2 h of heavy exercise (Hultman 1967 Hermansen Hultman and Saltin 1967)

5 h after completion of the exercise total glycogen had increased to 64 (45—89) mmol glucose units×kg [1] This value is similar to that found in the thigh at rest after con uming a mixed diet (Hultman 1967 Karlsson Diamant and Saltin 1971) After an additional 5 h it had ri en to 86 (72—101) mmol glucose units×kg [1] A further increa e was found during the remaining h but it was not as pronounced as that in the first 10 h of the experiment following completion of the exercise In the morning and late in the evening of the second day (22 and 34 h respectively after exercise) total glycogen was 95 (91—100) and (92—120 mmol glucose units×kg [1] and was 124 (11—131) mmol glucose units×kg [1] 46 h after initiation of the carbohydrate enriched diet The subject did not reach higher glycogen contents at the end of the experiment than was found at the start of the study This may be explained by the fact that all subjects were regularly physically active and consumed a carbohydrate rich diet

Evaluation of the glycogen distribution in the muscle samples was done on a total

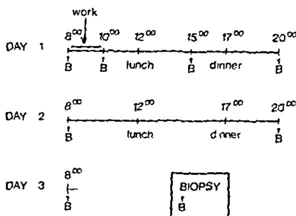



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individual muscle fibres after exercise induced glycogen depletion and to evaluate how this refilling of stores is correlated to carbohydrate intake Thus the caloric intake and its carbohydrate content was controlled and muscle biopsies were taken before and at given time interval after food intake It was found that about 60 % of the total increase in muscle glycogen content took place within 10 h after the exercise induced depletion During this period most of the intake of carbohydrate was found stored in the muscle The increase in glycogen content after exercise was more enhanced in the white than in the red fibres

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units×kg⁻¹ no difference in PAS staining intensity existed between the fibres However this does not preclude the existence of a difference in the glycogen content of the two fibre types since the intensity of the staining reaction is maximal before maximal glycogen concentrations are achieved The results of this study suggest that the critical muscle glycogen level where the PAS staining reaction can detect differences between fibres is between 80 and 100 mmol glucose units×kg⁻¹ (Fig 3)

## Discussion

Resynthesis of glycogen stores appears to start almost immediately after depletion A significant increase in muscle glycogen has been observed in rat muscle incubated with glucose after only 30 min (Adolfsson 1972) There are however data from human muscle indicating that no significant increase in muscle glycogen occurs following exercise until after 60 min (Karlsson and Saltin 1971)

In the first 5 h following depletion of glycogen stores approximately 800 mmol of glucose were supplied. This lead to an increase in total muscle glycogen of 41 mmol glucose units/kg⁻¹ During the rest of the period the muscle glycogen level continued to rise (Fig 2) but there was a marked discrepancy between total carbohydrate intake and calculated amount of glycogen stored in the muscle This is also apparent if the experimental period is divided into 24 h segments In the first 24 h approximately 2600 mmol glucose are supplied resulting in an increase in the musculature of 72 mmol glucose units×kg⁻¹ The same carbohydrate intake in the second 24 h period only produced an increase of 28 mmol glucose units/kg⁻¹ If a comparison is made between carbohydrate intake and glycogen stored in the muscle (based on 20 kg muscular mass) it appears that initially the entire carbohydrate intake was converted to muscle glycogen

A number of mechanisms may contribute to the rapid glycogen storage in the initial phase The low glycogen level in the cell is believed to have an affect on cell membrane permeability to glucose (Arvill 1967) and on the activity of the enzyme glycogen synthetase (Bergstrom *et al* 1972) One explanation for this is assumed to be that the glycogen in itself has an inhibitory effect on the enzyme which transforms the D form of glycogen synthetase into the I form thereby increasing activity in the redeposition phase (Villar Palasi and Larner 1960 When the glycogen level is reduced as a result of *e g* exercise this inhibitory effect declines and the activity of the I form increases However the significance of a low cell glycogen content can not provide the complete answer as present data suggest an equally rapid or perhaps faster rise in glycogen levels in FT fibres which were not as glycogen depleted after exercise as ST fibres Studies on rat and guinea pig have demonstrated that the red muscle fibres have a greater degree of synthetase activity Hess and Pearse 196[illegible] St George Stubbe and Blanchaer 1965 In human muscle [illegible] conflicting results have been reported Engel (1962) reported that type II fibre (perhaps fast twitch and St George Stubbe and Blanchaer 1965) that the red fibre (perhaps slow twitch) contain the highest activity of glycogen synthetase The time pattern [illegible] glycogen increase in the ST and FT fibres is similar but it might be somewhat f[illegible]

in the FT fibres of the human skeletal muscle This may be interpreted as an indication of a higher glycogen synthetase activity in the FT fibres as compared to the ST fibres

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Acta physiol scand 1974 90 303—318
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# Afferent Discharge from Human Muscle Spindles in Non Contracting Muscles Steady State Impulse Frequency as a Function of Joint Angle

By

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Received 12 April 1973

**Abstract**

Vallbo A B *Afferent discharge from human muscle spindles in non contracting muscles Steady state impulse frequency as a function of joint angle* Acta physiol scand 1974 *90* 303—318

Impulses in single muscle spindle afferents from the antebrachial finger flexor muscles were recorded from the median nerve of waking human subjects The discharge frequency in the steady state was measured as the metacarpo-phalangeal joint angles were passively changed in steps At a comfortable resting position of the hand less than ten percent of the spindle primaries were discharging Within intermediate ranges of muscle lengths the discharge frequency of the single units did not exceed 20 impulse per second (ips) The response to joint movements was that expected from muscle stretch and muscle release alone and there was no indication of fusimotor adjustments as a result of joint movement The frequency of the single units increased linearly with joint extension the mean sensitivity of the primaries being 0 18 ips per degree which is about 5 times lower than the sensitivity of de-efferented spindles in cat ankle extensors The afferent discharge from the secondaries was in most respects very similar to that of the primaries The low discharge frequency the low percentage of active units and the low position sensitivity all indicate an insignificant fusimotor outflow to relaxed human hand muscles and a comparatively poor ability of the spindles to measure muscle length under these conditions

The afferent discharge from muscle spindles is approximately linearly related to the muscle length in the steady state providing the fusimotor outflow is constant (for ref see Matthews 1964 1972 Haase and Ortgiese 1966 Granit 1970) The terms position response and position sensitivity have been coined to denote respectively the steady state discharge and the slope of the steady state discharge curve versus muscle length (Lennerstrand 1968 Lennerstrand and Thoden 1968) Although the matter is unsettled there is some indication that the fusimotor outflow may be reflexly modified by joint movements and/or muscle stretch (Hunt 1951 Eldred Granit and Merton 1953 Diete Spiff and Pascoe 1959 Fromm and Haase 1970 Proske and Lewis 1972) and a more complex relation between muscle length and spindle frequency may result when the innervation of the muscle is intact In any case it does

not seem possible to predict the relation between muscle length and spindle deschar ge from relaxed muscles in waking human subjects on the basis of data from anaest hetized decerebrate or spinal preparation On the other hand it seems an essential requisite for any functional evaluation of the spindle afferent discharge during na tural movements to define the resting discharge from relaxed muscles

The present study is an analysis of the steady state impulse frequency from muscle spindles in the antebrachial finger flexor muscles of waking human subjects at diffe rent mu cle lengths It was found that measures similar to position response and *position sensitivity* were u eful to describe the steady state impulse frequency as a function of joint angle indicating that to a first approximation, the impulse fre quency was a simple function of the muscle length The resting descharge was very insignificant when the hand was held in a comfortable resting position and the posi tion sensitivity was very low suggesting that the fusimotor outflow was negligible

## Methods

The present report is based upon analyses of activity from 64 muscle spindle afferents collected in 27 expts from 2? subjects aged between 19 and 26 years Single unit impulses were recorded from the right median nerve approximately 10 cm above the elbow with percutaneously inserted tungsten needle electrodes according to the method described in earlier reports (Vallbo and Hagbarth 1968 Vallbo 1970 1972) The endings were all located in the long finger flexor muscles as determined by their responses to local pressure passive joint movements and isomet ric oluntary contractions The subject was lying face down on a bed the upper arm extended laterally whereas the forearm rested on a firm support along the side of the body The forearm was pronated so that the palm was directed upwards The subject s hand was attached to a de nce which allowed the continuous measurement and recording of the metacarpo-phalangeal joint angles of the four ulnar fingers These fingers were fixed between two padded Perspex plates which kept them extended to 180 at the interphalangeal joints The finger plates could rotate around an axis which coincided with that of the metacarpo-phalangeal joints A potent ometer connected to the axis provided an analogue signal which was l nearl rela ed to the joint angles within the range of the study The wrist joint angle was slightly dorsiflexed as it was kept at 165 in most of the experiments In a few initial experiments this angle w s 135 There was no obvious difference between the data from these two sets of experiments. Angle calibrations were based upon measurements from the dorsal contours of the parts con idered i e the forearm hand and fingers

The tead state impulse frequency was determined as a function of the metacarpo-phalang al joint angle when this was changed in steps of 10 degrees 15 s after a passive joint movement the number of spike intervals were counted over ? s The number of observations fr m the indi idual units varied between 1 and 44 and the range of joint ar gle tested varied up to 40 Tl e recording electrode tended to be dislocated by larger joint movements The experi mental ar ar ement as well as the data collection system have been described in de ail in earlier repert Vallbo 1970 1972)

## Results

*Identificat on f ceptor type and selection of units*

The units were identified as muscle spindle afferents by means of a number of tests as described in an earlier report (Vallbo 1970) However it was found that on one point the basis of receptor identification needs to be modified It has been claimed that a poor relation between unitary impulse frequency and the torque due to active contraction under isometric conditions indicates that the ending is a spindle receptor

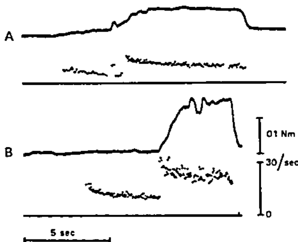



Fig 1 Responses of a Golgi tendon organ unit in the long finger flexor muscles to isometric voluntary contractions Upper traces torque due to contraction in metre newton (Nm) Lower traces instantaneous impulse frequency of the Golgi tendon organ afferent The receptor was related to the fourth finger and the test contractions were isometric flexions of this finger

and not a Golgi tendon organ as the tendon orgnas are supposed to provide continuously an accurate signal of the active force of the contracting muscle (Jansen and Rudjord 1964 Houk and Henneman 1967) However it was found in the present study that the tendon organ discharge may be a discontinuous function of the active force An example is given in Fig 1 which shows respon es in a tendon organ afferent to isometric voluntary contractions It may be seen that the discharge took up one of three rather distinct frequency levels of 5—8 ips 9—12 ips and 20—25 ips represented respectively in A A and B and in B The steps from one level to the other were abrupt whereas the force output of the muscle was mostly changing smoothly It may also be seen that the discharge at these frequency levels was time dependent declining monotonically and at a decreasing rate These characteristics are very similar to those described by Houk and Henneman (1967) for Golgi tendon organs in the cat when single skeletomotor nerve fibres were stimulated tetanically one in isolation and two or three in combinations Thus the discharge presented in Fig 1 is most reasonably interpreted as responses from a Golgi tendon organ when one two or three motor units inserting onto the receptor were recruited during the voluntary contractions On the other hand it seems difficult to explain the discharge as muscle spindle responses on the basis of the present knowledge concerning the fusimotor effects on spindle afferent discharge particularly as revealed in frequencygrams (Bessou Laporte and Pages 1968) With regard to the problem of identification of receptor type it seems relevant that the responses shown in Fig 1 may give rise to a poor torque frequency relation and this may therefore not be taken as a clear indication of spindle origin of the discharge

An important basis for classifying a receptor as a muscle spindle in contrast to a Golgi tendom organ in the present study was a continuous discharge when the muscles were relaxed (Matthews 1933 Jansen and Rudjord 1964 Alnæs 1967 Houk and Henneman 1967 Houk Singer and Henneman 1971 Stuart *et al* 1970) Consequ

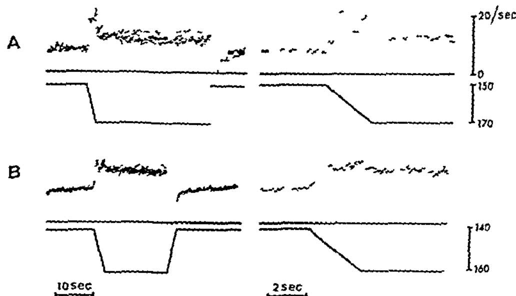



Fig 2 Spindle afferent responses to passive joint movements in relaxed muscles Upper traces instantaneous impulse frequency Lower traces metacarpophalangeal joint angles A and B show discharges from 2 separate spindle afferents both related to the fourth finger To the right the stretch phases are displayed on an expanded time scale

ently there was very likely an over representation of spindle afferents which were spontaneously firing at intermediate muscle lengths This was particularly the case for the secondary endings as they were to a large extent identified on this ground whereas for the primary endings also a number of other tests were helpful. This likely selection is probably relevant for some quantitative aspects of the present findings as will be considered below

*Spindle response to muscle stretch*

When the muscle was stretched by passive joint movements the spindle discharge increased as shown by the examples of Fig 2 Some units exhibited a pronounced dynamic response as in Fig 2 A whereas the dynamic response was small or negligible for other units as in Fig 2 B The endings were defined as primary and secondary endings respectively on the basis of their dynamic index (Crowe and Matthews 1964) estimated from recordings of their responses to passive muscle stretch as in Fig 2 For most units this classification was unequivocal Only a few endings exhibited intermediate dynamic responses and thus were classified on a somewhat arbitrary basis Of the total 64 spindle afferents studied 43 were primaries and 21 were secondaries After completion of the stretch the impulse frequency declined slowly at a decreasing rate and attained a reasonably steady state within 5—15 s The frequency then remained constant for as long as it was observed which was up to

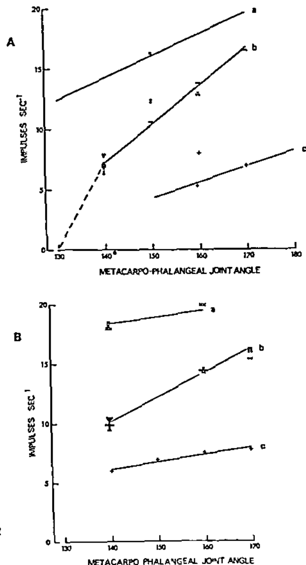



Fig 3 Steady state activity of 6 spindle afferents as a function of joint angle in relaxed muscles Data from 3 primaries are shown in A and data from 3 secondaries in B The symbols represent the experimental data whereas the fulldrawn lines represent linear equations fitted to the data by means of the method of least squares The regression coefficients ($k$) and the correlation coefficients ($r$) were as follows A a $k = 0\ 18$ $r = 0\ 93$ b $k = 0\ 32$ $r = 0\ 95$ c $k = 0\ 13$ $r = 0\ 82$ B a $k = 0\ 06$ $r = 0\ 76$ b $k = 0\ 21$ $r = 0\ 97$ c $k = 0\ 06$ $r$ 0 98

minutes Non monotonic changes of the frequency after the end of the stretch were not seen A stretch of the muscle was always associated with a rise of the steady state discharge and *vice versa* There was only one exception for one unit which was studied in only one test the firing rate just decreased for an extension of ten degrees

The steady state impulse frequencies as a function of the joint angle are shown for 6 units in Fig 3 3 primaries in A and 3 secondaries in B The symbols represent the experimental findings The plots suggest a linear relation between joint angle and steady state discharge The same was true for the other units However the linear

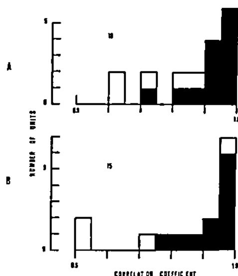


Fig 4

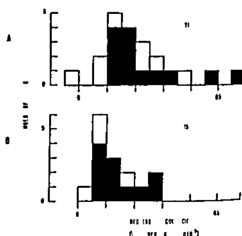


Fig 5

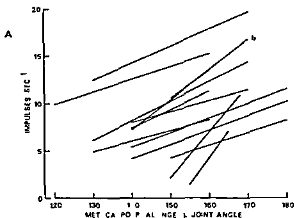
Fig 4 Histograms showing the distribution of correlation coefficients between steady state discharge and metacarpo phalangeal joint angle for primaries in A and secondaries in B. Black indicates $P < 0.05$ and white $P > 0.05$

Fig 5 Histograms showing the distribution of regression coefficients of muscle spindle steady state discharge on joint angle. Data from primaries are shown in A and data from secondaries in B. Statistical significance of the correlation between steady state frequency and metacarpo-phalangeal joint angle is indicated as in Fig 4

range obviously did not extend below 3—5 ips. An example is shown by the data marked *b* in Fig 3 A. The phenomenon simply indicates that continuous firing does not occur at these low frequencies, as is well known from animal experiments. The finding has, however, some particular relevance in the present study as will be considered below. Apart from this deviation from linearity no other discontinuity was found in the steady state discharge as a function of joint angle. The variability as shown in the plots of Fig 3 is representative. The variation in impulse frequency at the same joint angle for one and the same unit was usually 2—4 ips. This seems remarkably high, at least when put in relation to the absolute frequency covered a range of 5—20 ips. However, to adequately appreciate the reproduceability and the consistency of the steady frequency it should be recognised that the measurements were done after muscle stretch as well as after muscle release and further that the data from some units were extracted at several occasions during long lasting experiments. In between a number of other procedures were undertaken, such as isometric and isotonic voluntary contractions.

Linear equations were fitted to the experimental data from the individual units by the method of least squares and the correlation coefficients of the steady state impulse frequency on joint angle were calculated. The full drawn lines in Fig 3 represent the equations for which the regression coefficients ($k$) are given in the legend, as are also the correlation coefficients ($r$). Data from 33 units or 52 % of the

A




B

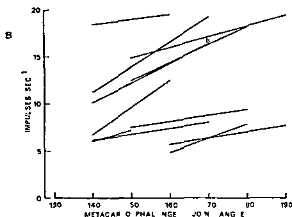



Fig 6 Diagram showing the relation between steady state impulse frequency and metacarpo-phalangeal joint angles for primaries in A and secondaries in B The lines represent linear equations fitted to the experimental data from the individual units The correlation coefficients were statistically significant ($P < 0.05$) for all the units included a b and c indicate the units which are also illustrated in Fig 3 whereas the asterisks indicate units which were studied at a slightly different position of the wrist joint implying that the muscles were a bit longer at corresponding abscissa values compared to the other ones

total sample allowed a quantitative analysis of this type The remaining 58 % were silent at all angles tested or too few observations were available altogether The correlation coefficient was statistically significant ($P < 0.05$) for approximately 70 % of the units analysed (24/33) as shown by the sample histograms of Fig 4 where primaries are represented in A and secondaries in B Filled blocks indicate significant correlations and white blocks indicate significant correlations Non significant correlations were found largely when very few observations were available for any one unit whereas the correlations were in fact significant for 90 % of the units from which ten observations or more were available There was no obvious difference between the primaries and the secondaries with regard to the correlations as may be appreciated from Fig 4 The findings indicate that the muscle spindle discharge increased approximately linearly with the joint extension Hence it seems reasonable to employ the concept of position sensitivity defined as the number of impulses per second per degree joint movement at the metacarpo phalangeal joints

The position sensitivities are given by the regression coefficients which are presented in the histograms of Fig 5 for the 33 units of Fig 4 and for 3 additional units

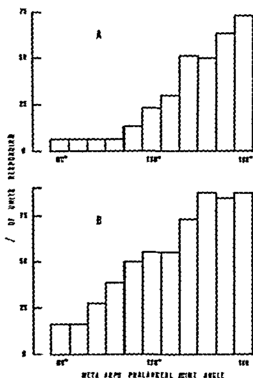



Fig 7 Diagrams showing the percentage of units which were continuously discharging in the steady state as a function of the metacarpo-phalangeal joint angle Data from the primaries are displayed in A and data from secondaries in B The diagrams are constructed on the basis of the linear equations shown in Fig 6 extrapolated down to 3 ips and experimental data concerning the number of non-discharging units encountered at an joint angle

These 3 units were not included in Fig 4 as only two observations from the individual unit were available Statistical significance is indicated by filled blocks. It may be seen that for the majority of the units the position sensitivities varied between 0 05 ips/degree and 0 30 ips/degree The two very high values are probably fallacious as many of the data from these two units were located in the low range where the frequency falls off rapidly The one negative value on the other hand was based upon only two observations and it seems therefore not to merit too much attention (cf Haase and Ortgiese 1966 Apart from these 3 units there was a remarkable uniformity in the samples The means of the regression coefficients were 0 184 ips/degree for the primaries and 0 143 ips/degree for the secondaries For these calculations the data were excluded from the units which exhibited non-significant correlations as well as the two values above 0 4 ips/degree Including these two values would raise the mean for the primaries to 0 237 ips/degree The difference between the two samples from the primaries and the secondaries was not statistically significant (P > 0 05 Komolgorov-Smirnov test The muscle spindle sensitivity in units of ips/mm muscle length change may be estimated on the basis that a 10 flexion at the metacarpophalangeal joint gives rise to a muscle shortening of 2—3 mm (Landsmeer 1955 Kaplan 1965 Thus the position sensitivity of the primaries would be 0 6—0 9 ips/mm

All the units for which significant correlations were found are presented in Fig 6 as straight lines fitted to the experimental points with the method of least squares

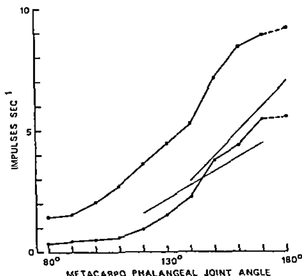



Fig. 8. Diagram showing the average steady state frequency in spindle afferents as a function of joint angle. Squares and upper curve refer to secondaries and filled circles and lower curve to primaries. The curves are constructed on the basis of the total single unit sample including discharging as well as non-discharging units. The thin lines represent linear equations fitted to data from two multi-unit recordings scaled to match approximately the frequency of the single unit sample curve for the primaries.

Primary endings are shown in A and secondary endings in B. The curves marked *a*, *b* and *c* refer to the units of Fig. 3, whereas the curves marked with an asterisk refer to the units studied when the wrist joint angle was slightly more extended (155°) than it was in the majority of the experiments (165°). The diagrams of Fig. 6 display the range of joint angles over which the units were studied, the frequency of their discharge as well as the position sensitivities for the most comprehensively analysed units. It is obvious that the study covers a small range of muscle length changes compared to the maximal ones which might occur in the body; 60° at the metacarpo-phalangeal joints would give rise to less than 25% of the maximal muscle length change (Kaplan 1965). Further, it is obvious that only an intermediate range of muscle lengths is covered as the wrist joint was some 30° short of maximal extension. An implication is, of course, that the linear relation between steady state impulse frequency and the joint angle may not apply equally well over the whole range of movements. In addition to the units presented in Fig. 6, there were a number of non-discharging units at any joint angle tested, so the inter-unit variation at the two extreme joint positions of Fig. 6 ranged from no discharge to approximately 10 ips and 20 ips respectively. No difference was found between primaries and secondaries with regard to the impulse frequency, as may be appreciated from Fig. 6. It may also be seen in Fig. 6 A that the 2 curves with the highest slopes are to a great extent located in the low frequency range where the discharge tends to fall off abruptly. This seems to justify the exclusion of these data from the calculation of the mean position sensitivity as discussed above.

*Recruitment of spindle endings*

The units varied considerably with regard to their thresholds for a steady state discharge in terms of joint position. Hence more and more spindle afferents were

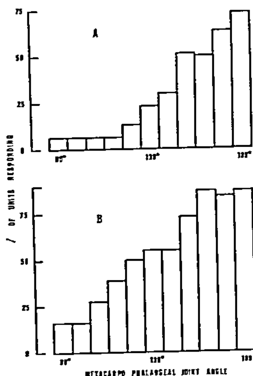



Fig 7 Diagrams showing the percentage of units which were continuously discharging in the steady state as a function of the meta carpo-phalangeal joint angle Data from the primaries are displayed in A and data from secondaries in B The diagrams are constructed on the basis of the linear equations shown in Fig 6 extrapolated down to 3 ips and experimental data concerning the number of non-discharging units encountered at any joint angl

These 3 units were not included in Fig 4 as only two observations from the individual unit were available Statistical significance is indicated by filled blocks It may be seen that for the majority of the units the position sensitivities varied between 0 05 ips/degree and 0 30 ips/degree The two very high values are probably fallacious as many of the data from these two units were located in the low range where the frequency falls off rapidly The one negative value on the other hand was based upon only two observations and it seems therefore not to merit too much attention (*cf* Haase and Ortgiese 1966) Apart from these 3 units there was a remarkable uniformity in the samples The means of the regression coefficients were 0 184 ips/degree for the primaries and 0 143 ips/degree for the secondaries For these calculations the data were excluded from the units which exhibited non-significant correlations as well as the two values above 0 4 ips/degree Including these two values would raise the mean for the primaries to 0 237 ips/degree The difference between the two samples from the primaries and the secondaries was not statistically significant ($P >$ 0 05 Komolgorov-Smirnov test) The muscle spindle sensitivity in units of ips/mm muscle length change may be estimated on the basis that a 10° flexion at the meta carpophalangeal joint gives rise to a muscle hortening of 2—3 mm (Landsmeer 1955 Kaplan 1965 Thus the position sensitivity of the primaries would be 0 6—0 9 ips/mm

All the units for which significant correlations were found are presented in Fig 6 as straight lines fitted to the experimental points with the method of least squares

were searched The total number of impulses from a group of muscle receptors was counted in the steady state in 2 expts The contributing afferents were all assumed to originate from muscle spindles as very few other receptors in the muscle discharge continuously in the steady state under these conditions Linear equations were fitted to the data as described previously for the single units In both cases highly significant correlations were found ($r = 0.960$ $P < 0.001$ $r = 0.787$ $P < 0.001$) Obviously the number of units contributing to the total discharge was not known in these recordings It was therefore not possible to calculate properly the position response and position sensitivity of the average single unit to be compared with the single unit data However if the multi unit data were scaled to match approximately the average spindle discharge frequency as determined from the single unit sample the slopes of the curves would match The fine straight lines in Fig 8 were obtained for the two multi unit recordings It may be seen that the slopes agree reasonably well with that of the lower curve within the appropriate range of joint angles the regression coefficients being 0 108 ips/degree and 0 070 ips/degree for the 2 sets of multi unit data against 0 099 ips/degree for the curve constructed on the basis of the single unit sample of the primaries The scaling involved the assumption that the number of units contributing was 8 and 10 respectively in the 2 expts

## Discussion

It was shown in the present study that the steady state discharge from human muscle spindles was a simple function of the joint angle in waking subjects when the muscles were relaxed After a joint movement the spindle discharge declined or increased monotonically at a decreasing rate and there were no indications of fusimotor adjustments as a result of the joint movements Although the findings are most simply explained as accounted for by the muscle length changes alone it cannot be excluded that small and smooth changes of the fusimotor outflow might have occurred It was obvious from inspection of instantaneous impulse frequency records that the discharge attained a steady state within 5—15 s after a joint movement The very slow change in spindle frequency after a movement was not analysed however and some further small decrease of the discharge with time might occur particularly in afferents from primary endings (Lennerstrand 1968 Poppele and Bowman 1970) An implication of measuring the discharge at 15 s after joint movements is that hysteresis might give rise to some scatter of the intra individual data Primaries are known to have a more pronounced hysteresis than secondaries in cat de efferented spindles when the frequency is measured at this time after a change in muscle length (Lennerstrand 1968) In the present study the scatter was not markedly different for primary endings and secondary endings as may be appreciated from the correlation coefficients suggesting that hysteresis was not all that significant Part of the scatter might be accounted for by changes in the mechanical links between the spindle and the angle recording device

The human hand attains a resting position which is remarkably determinate, as appreciated introspectively, when the muscles are relaxed and the hand and the fore arm are leaning on their ulnar aspects against a firm support the wrist joint is slightly dorsiflexed (approximately 160°) whereas the finger joints are intermediately flexed (130°—150°) In the present study this particular position was not employed but an equivalent position, with regard to the length of the long finger flexor muscles was probably attained in the present experiments when the metacarpo phalangeal joint angles were 110° and the interphalangeal joints were fully extended This estimation is based upon the assumption that an extension of the interphalangeal joints by 30°—50° would be approximately compensated for by a flexion of the metacarpo phalangeal joints by 20°—40° with regard to the muscle lengths (Landsmeer 1955) Although this evaluation is somewhat arbitrary it seems that the error would not be too large and a small error would not make too much difference to the following reasoning The findings indicate that the number of active primaries and the average discharge frequency from the primaries are almost minimal in the comfortable resting position of the human hand Any further decrease of the muscle lengths would have very small effects whereas any increase of the muscle lengths from this position would increase the two considerably Similar findings have been reported earlier for hand muscles (Vallbo 1970) and recently also for leg muscles (Hagbarth Wallin and Lofstedt 1973) The percentage of active primary afferents at the comfortable resting position would be below ten and the total discharge from the approximately 700 spindle primaries in the antebrachial finger flexor muscles to the 4 ulnar fingers (Voss 1959) would be below 500 ips which is not more than the discharge provided by four single end organs in a strong contraction (Vallbo 1973) Whether the same or somewhat higher values are valid for the secondaries as well cannot be safely assessed on the basis of the present data but it seems likely that there is not much difference between human primaries and secondaries in this respect Sampling bias very likely accounted for at least some of the observed difference between the two types of afferents Further consistent differences have not been found in studies on animals between the two types of endings with regard to threshold and discharge frequency as a function of muscle length (Hunt 1954 Diete Spiff 1961 Harvey and Matthews 1961 Bessou and Laporte 1962 Fehr 1962 Renkin and Vallbo 1964 Alnaes Jansen and Rudjord 1965)

The spindle discharge was weaker than expected from studies in the cat with regard to the impulse frequency of the individual units and with regard to the proportion of spontaneously discharging units For an adequate comparison between cat and human spindle characteristics it would be desirable to know the muscle lengths at different joint angles in relation to the maximal length and zero length i e the length when there is no tension at the tendon These data were not available but it seems possible to define an upper limit for the spindle discharge at zero muscle length to be compared with similar data from animal experiment by adopting certain assumptions It seems reasonable to assume that the antebrachial finger flexor muscles are close to their zero length at the comfortable resting position of the human

hand If anything they are probably stretched a little beyond this length as the resting position of the hand is presumably determined largely by the balance between the forces exerted by the passive flexor and extensor muscles On this basis the present data may be compared with findings from animal experiments In cat hind limb extensors which have been the object of most animal studies in this field virtually all the spindle primaries are firing continuously at zero length even when the spindles are de efferented However in other cat muscles many primaries are silent and in the rabbit most of the primaries are silent under similar conditions to judge from the few data available (Hunt 1954 Diete Spiff 1961 Harvey and Matthews 1961 Bessou and Laporte 1962 Fehr 1962 Alnæs Jansen and Rudjord 1965) Thus there is definitely a difference between cat hind limb extensor spindles and human finger flexor muscle spindles The difference cannot be accounted for by central mechanisms as the human spindle discharge was less than the de-efferented cat spindle discharge The possibility that the difference is related to the fact that ankle extensors have a prominent role in posture in contrast to the human finger flexors is not supported by the experience on human ankle extensor muscle spindles as their discharge did not seem to be very prominent either (Hagbarth and Vallbo 1968 1969 Hagbarth Wallin and Lofstedt 1973) Thus the present findings indicate that there may be pronounced differences between species in this respect although the functional implications cannot be assessed at present

Another striking property of the human spindles was their low sensitivity to joint position compared to the cat hind limb extensor spindles It was found that the sensitivity of the primaries was 0 18 ips/degree at the metacarpo phalangeal joint whereas the sensitivity of the cat spindles is about 1 0 ips/degree at the ankle joint *i e* about 5 times higher This figure has been deduced from the position sensitivity of de efferented primaries and primaries with very little fusimotor drive in the cat hind limb extensor which is approximately 4 0 ips/mm (Jansen and Matthews 1962 Alnæs Jansen and Rudjord 1965 Lennerstrand 1968 Brown Lawrence and Matthews 1969) and from the finding that one mm length change of the ankle extensor muscles corresponds to approximately 4° movement of the ankle joint (Rack and Westbury 1969 Houk Singer and Henneman 1971) A similar difference exists if the position sensitivity is expressed in units of ips/mm as the human finger flexor spindle primaries have a position sensitivity in the order of magnitude of 0 6—0 9 ips/mm against 4 0 ips/mm for the cat ankle extensor spindles Thus the human finger flexor spindles are about 5 times less sensitive regardless of which definition sensitivity is given A simple basis for the difference between the two types of muscles may be provided by assuming that the spindle response to any given absolute change in muscle length is larger for small muscles simply because the proportional change in muscle length is larger If this effect is not fully compensated for *e g* by the longer muscles having proportionally longer spindles it seems reasonable that the position sensitivity would be inversely related to the muscle length (*cf* Andersson Lennerstrand and Thoden 1968)

The present data indicate that the fusimotor outflow to the human finger flexor

muscles was insignificant, as the discharge from the spindles as a group was very weak when the hand was held in a comfortable resting position and the muscles were relaxed Blocking of the small nerve fibres with lidocaine also indicates that there is a little or no fusimotor outflow to relaxed human muscles (Hagbarth Hongell and Wallin 1970 Wallin Hongell and Hagbarth 1973) —However it does not seem safe to claim that there was no fusimotor outflow at all and that is for two reasons First the relation between lengths and spindle frequency might be so different in man and cat that the fusimotor activity in man cannot be appreciated from a comparison between the position responses in the two preparations Second there was a pronounced irregularity in some unitary discharges as to suggest a static fusimotor drive (Bessou Laporte and Pages 1968 Matthews and Stein 1969 a) However, from the functional point of view the significant aspect is that over a considerable range of muscle lengths the spindle discharge was so small and it was so insensitive to muscle length that the spindles can have virtually no function at all It seems that this is valid at least for muscle lengths below those attained in the comfortable resting position of the hand when the muscles are relaxed

It should be noticed that the range of muscle lengths over which the spindle afferents were studied in the present analysis covers a very important working range of the human hand in delicate manual work In this range it was found that the rate of spindle recruitment on passive muscle stretch was relatively high and the sensitivity of the spindles as a group was higher than it was at shorter muscle lengths. On the basis of spindle counts in human muscles the total response of the spindles in the long finger flexor muscles to passive movements may be calculated The superficial and the deep flexor muscles contain together 682 spindles (Voss 1969) If it is assumed that 25 % of them respond to the movement of a single finger it follows that the change in total impulse discharge per degree at the metacarpo phalangeal joint would be only 16 8 ips as one finger is passibely moved The response from the secondaries would be a little more or 25 2 ips on the assumption that the number of secondaries per spindle is 1 5 (Barker 1962 Boyd 1962 Boyd and Davey 1968 Kennedy 1970) Thus it seems that the set of first order neurones taken together provide a signal from which the muscle length cannot be accurately deduced in the steady state unless there is an extremely high convergence onto higher order neurones The fact that spindle sensitivity is actually much higher for small changes in muscle length (Matthews and Stein 1969 b) does not invalidate this conclusion as this property does not help to evaluate the absolute muscle length but rather to signal a change in muscle length

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From the Department of Physiology University of Umeå Sweden

# Human Muscle Spindle Discharge during Isometric Voluntary Contractions Amplitude Relations between Spindle Frequency and Torque

By

A B VALLBO

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**Abstract**

VALLBO A B *Human muscle spindle discharge during isometric voluntary contractions Amplitude relations between spindle frequency and torque* Acta psysiol scand 1974 *90* 319—336

During isometric voluntary contractions the impulse frequency in muscle spindle afferents was analysed in relation to the contraction intensity in man Single unit impulses from the finger flexor muscles were recorded with percutaneously inserted tungsten needle electrodes from the median nerve The spindle frequency increased with the contraction intensity For very weak contractions the increase was steep but levelled off to attain an average slope of 32 8 ips/Nm at the metacarpo-phalangeal joint for the primaries and 22 8 ips/Nm for the secondaries when the muscles to a single finger were activated The close relation between spindle frequency and torque suggests parallel modulations of the skeletomotor and the fusimotor outputs during voluntary contractions The dominating type of fusimotor outflow was of the static type but clear indications of increased dynamic fusimotor outflow were also seen Quantitative considerations of the spindle frequency as a function of contraction intensity and as a function of muscle length suggest that the muscle spindles and their central connections do not constitute a very powerful mechanism for holding the muscle at constant length when the load varies

It seems that a knowledge of the qualitative and quantitative properties of the afferent discharge from muscle spindles during natural contractions is essential for an understanding of their functional role This type of data are virtually equivalent to the basic stimulus response characteristics of simpler receptors when considering the function of the sense organ However an analysis of the muscle spindle response characteristics as they would be in the intact organism is considerably more complicated than in simpler receptors because of the double input mechanical and fusimotor A natural fusimotor input associated with any motor situation requires that the relevant central mechanisms are functionally intact which may be difficult to ensure in many animal experiments but it is obviously the case in voluntary contractions of waking human subjects It has been shown that such contractions are associated with a fusimotor outflow which is powerful enough to raise the afferent

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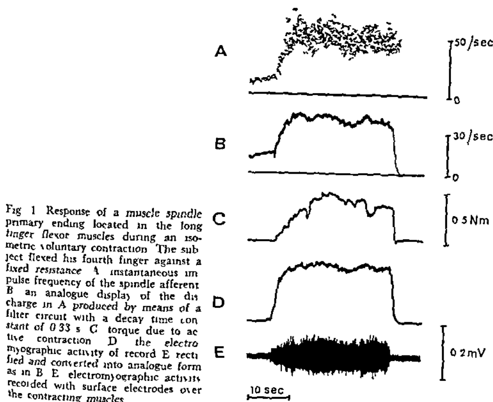



Fig. 1. Response of a muscle spindle primary ending located in the long finger flexor muscles during an isometric voluntary contraction. The subject flexed his fourth finger against a fixed resistance. A instantaneous impulse frequency of the spindle afferent. B an analogue display of the discharge in A produced by means of a filter circuit with a decay time constant of 0.33 s. C torque due to active contraction. D the electromyographic activity of record E rectified and converted into analogue form as in B. E electromyographic activity recorded with surface electrodes over the contracting muscles.

experiment. The data were accordingly extracted and analysed in blocks, one block being constituted of the data from one or several contractions performed at the same joint angle and in succession without intervening procedures except for short resting periods. The number of data blocks at one and the same joint angle varied between one and three for the individual unit. The total number of blocks was 69. Analogue signals of the nerve discharge frequency and the intensity of the electromyographic activity were occasionally produced by means of an active filter circuit with a variable decay time constant (Fig. 1 and 10). Before the original signals were integrated in this way the electromyographic activity was full wave rectified and the nerve impulses were transformed into unit pulses of suitable size and shape.

The total number of observations was 1854. As the data extracted over one second constitute one observation it follows that the total number of observations cover a time period of approximately thirty minutes. The number of observations from the individual unit, on the other hand, varied between 14 and 223. The experimental data were taped and processed afterwards.

## Results

Afferent activity from a muscle spindle primary ending during an isometric contraction is shown in Fig. 1. The subject flexed his fourth finger against a fixed resistance. In A the spindle discharge is displayed as the instantaneous impulse frequency and in B the same activity is shown in an analogue form. The decay time constant of the integration circuit employed was 0.33 s. The torque due to active contraction is

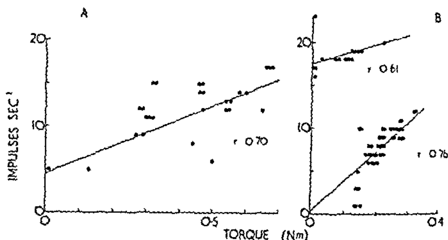



Fig 2 Impulse frequencies from a muscle spindle primary ending in A and two secondary endings in B plotted against the torque due to isometric voluntary contractions of the index finger flexor muscles The points represent the experimental data and the lines represent linear equations fitted by the method of least squares The correlation coefficients (r) are given in the diagrams Sampling period length was 1 0 s and sampling interval 2 0 s

shown in C and the associated electromyographic activity recorded with surface electrodes in E This signal was rectified and integrated with a decay time constant of 0 33 s in order to produce the analogue signal of the electromyographic activity displayed in D From a comparison of the signals in B C and D it may be appreciated that the spindle discharge frequency was closely related to the intensity of the skeletomotor contraction whether this was measured as torque (Fig 1 C) or as integrated electromyographic activity (Fig 1 D) In this particular test the agreement was more pronounced between spindle frequency and integrated electromyographic activity than it was between spindle frequency and torque This was not always the case but the reverse was also seen The slow rise in spindle frequency before the contraction started in the test of Fig 1 represents a recovery after the depressant effect of the preceding contraction which was performed some fifteen seconds before It is also apparent from Fig 1 A that the spindle discharge was very irregular indicating that the fusimotor activity accounting for the increase in frequency was to a great extent of the static type (Bessou Laporte and Pages 1968 Matthews and Stein 1969) The record is qualitatively representative of the majority of the spindle primary endings during isometric contractions in this respect

Quantitative data were extracted from recordings of this type by counting the number of impulses and measuring the average torque over 1 s every other second continuously during the contraction The points in Fig 2 plot data from a primary ending in A and two secondary endings in B It is evident that there was a positive relation between torque and spindle frequency although the scatter was pronounced For many units a plot of the type shown in Fig 2 suggested a linear relation for others the points rather seemed to fall along a negatively accelerating curve at least in the lower range whereas for still others the data were too scant to judge For all

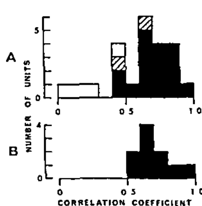



Fig 3

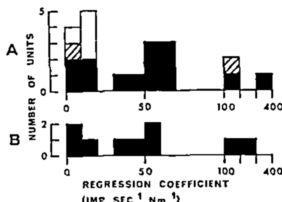



Fig 4

Fig 3 Histograms sh wing the distribution of correlation coefficients between muscle spindle impulse frequency and torque due to i ometric voluntary contraction for single spindle af ferents A data from 23 primaries B data from 10 secondaries Black indicates P < 0 01 hatching P < 0 05 and white P > 0 05

Fig 4 Histograms showing the distribution of regression coefficients of muscle spindle impulse frequency on torque due to isometric voluntary contraction for single spindle afferents The endings were all located in the long finger flexor muscles A data from 20 primaries B data from 9 secondaries Significance levels of the correlation between the two variables are indicat ed as in Fig 3 Note that the scale is compressed in the right hand part of the diagrams

the units there was a considerable scatter and it seemed difficult to define with confidence the exact form of the relation between torque and spindle frequency from the collected data However as a reasonable approximation and in order to achieve data reduction linear equations were fitted to the data from the individual units and the coefficients of product moment correlation were calculated in order to provide an estimate of the straight line fit There was a considerable inter individual variation as well as intra individual variation the latter being the variation blocks of data extracted from the same units (see Methods) The units may be separated in three groups with regard to the correlation between torque and frequency For 55 % of them a statistically significant correlation (P < 0 05) was present in all the avail able data blocks whereas the correlation was significant in some but not all the available data blocks for 33 % of them In the remaining 12 % of the units the cor relations were insignificant throughout In order to describe the frequency torque relation only the best data block from the individual unit was considered *i e* the block which exhibited the highest significance When not otherwise stated the data to be presented in the following refer to these selected data blocks

The correlation coefficients are shown in the histograms of Fig 3 from all the 33 units analysed Primaries are represented in A and secondaries in B For all the econdary endings and for 83 % of the primary endings the correlation was statistical ly significant in the best data block (P < 0 05) The probability levels are indicated by black hatching and white representing respectively a probability below 0 01 below 0 05 and above 0 05

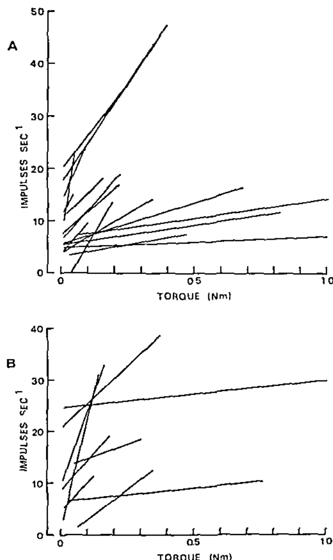



Fig 5 Diagrams showing the relations between impulse frequency and torque due to isometric voluntary contractions for single spindle endings located in the long finger flexor muscles The lines represent linear equations fitted to the experimental data A data from 16 primaries B data from 9 secondaries The extension of the lines agrees with the range of experimental data except for 3 units which were tested at torques above 1 Nm The correlation between impulse frequency and torque was significant ($P < 0\ 05$) for all the data blocks which constitute the bases of the diagrams

The spindle discharges illustrated in Fig 2 increased by approximately 1 5 1 0 and 3 5 ips (impulses per second) respectively for an increase of the torque by 0 1 Nm This is approximately the torque required to hold 100 g weight at the finger tip The corresponding figure varied considerably from one spindle to the other a may be seen in the histograms of Fig 4 showing the regression coefficients for the finger flexor primaries in A and secondaries in B Again black hatched and white areas indicate the probability level as in Fig 3 Note that the abscissa is compressed above one hundred The regression coefficients varied approximately with a factor of 100 which seems as a remarkably wide range However there were some indications that the values above 100 ips/Nm may be accounted for by a particular factor as will be considered below If these values are excluded the range of the

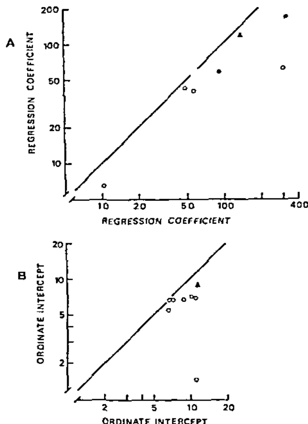



Fig 6 Diagrams to illustrate consistency of the relation between impulse frequency and torque within pairs of data blocks extracted from the same units. The regression coefficients (A) and the ordinate intercept (B) of one data block within the pair were plotted against that of the other. Circles represent primaries and triangles secondaries. Open circles indicate that the two data blocks were extracted at the same joint angle, filled symbols that the two sets of data were extracted at two joint positions differing by 10° at the metacarpo-phalangeal joint. The lines which represent X = Y are drawn in order to facilitate comparison between the 2 constants from a pair. The fact that all the symbols are located below the lines has no significance. Logarithmic scales were employed in order to compress the diagrams. The plots are based upon all the available data blocks for which the correlation coefficients were significant ($P < 0.05$)

regression coefficient would be brought down to approximately 20%, *i.e.* from 375 ips/Nm to 70 ips/Nm

Linear equations were fitted to the experimental data from 25 finger flexor units for which the correlations were significant. Diagrams of the equations are shown in Fig 5 where primaries are represented in A and secondaries in B. The extensions of the lines correspond to the ranges of contraction intensities tested with the exception of 3 units for which some data were available between 1 and 2 metre newton. In order to display more clearly the findings within the lower range of torque the pieces of the lines beyond 1 Nm were sacrificed for these 3 units. It is obvious that most of the units were studied over a relatively narrow range as the maximal contraction of *e.g.* the index provides a torque in the order of magnitude of 4 Nm (Vallbo 1970a). The maximal frequency in the sample did not exceed 50 ips and it was for some units below 10 even at relatively strong contractions.

The diagrams of Fig 5 are based upon one data block from each unit. In order to illustrate some properties of the intra-individual variation, pairs of data blocks from the same units were compared. Data blocks with non-significant correlations ($P > 0.05$) were, however, discarded from this analysis. In Fig 6 A the regression

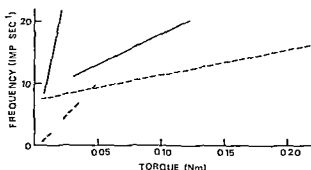



Fig 7 Diagrams to demonstrate difference between weak and strong contractions with regard to the slope of the relation between impulse frequency in single spindle afferents and the torque due to isometric voluntary contractions The lines represent linear equations fitted to two pairs of data blocks from two primary endings Full drawn lines and interrupted lines refer to the two units respectively The extension of the lines agree with the range of the experimental data The regression coefficients for the 4 equations were 1033 ips/Nm 98 ips/Nm 219 ips/Nm and 41 ips/Nm respectively

coefficient of one block within the pair is plotted against that of the other whereas Fig 6 B shows the same type of plot for the ordinate intercept The significance of the symbols is given in the legend It may be seen that there was a clear consistency between the two data blocks within the pairs for most of the units indicating that the frequency torque relation was similar for the individual unit when this was studied at different occasions during an experiment

It is obvious from Fig 3 that the correlation between frequency and torque was not very high for most of the units This was largely accounted for by a seemingly random scatter of the data points above and below the line representing the linear equation However for some units there seemed to be a systematic deviation from linearity particularly in the lower range where the impulse frequency increased more steeply Examples are shown in Fig 7 Linear equations were fitted to 2 pairs of data blocks from two different units One data block from each unit was extracted largely from weak contractions and the other one mainly from stronger contractions The full drawn lines and the dashed lines refer to the two different units respectively The extensions of the lines correspond to the ranges of torque of the individual data block It is obvious that the spindle discharge frequency increased much steeper with torque at weak contractions than it did at stronger contractions the regression coefficients were 5 to 10 times higher for the small contractions Although this could not be demonstrated clearly for more than a couple of units there were some indications that it was valid for most of them as the ordinate intercepts for most of the equations were higher than the actual unitary discharge when the muscles were non contracting For instance the resting discharge was zero for 14 of the 25 units illustrated in Fig 5 whereas the ordinate intercept was above zero for all but one of them Further when a resting discharge was present the ordinate intercept was usually higher as shown in Fig 8 On the other hand the ordinate intercept of the equations fitted to the weak contraction data as shown in Fig 7 was close to zero These two units were non discharging when the muscles were relaxed

Thus there was very likely a systematic non linearity in this range One implica

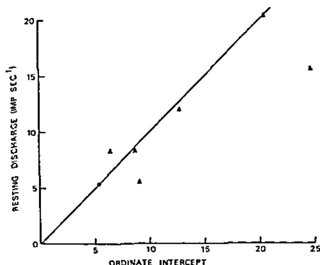



Fig 8 Diagrams showing the relations between resting discharge of single spindle afferents and ordinate intercept of the linear equations fitted to the experimental data of impulse frequency as a function of torque due to isometric voluntary contractions for the same units Filled circles indicate primaries and triangles secondaries The line which represents X = Y is drawn to facilitate the comparison between the two variables

tion of this finding is that the very high regression coefficients as were found in 5 units (Fig 4) might be accounted for by very weak contractions An inspection of Fig 5 actually indicates that the 5 regression coefficients above 100 were all obtained from data which did not extend beyond 0 2 Nm Thus it seems justified to reject the regression coefficients above 100 when considering a wider range of contraction intensities

*Average spindle frequency as a function of torque* The units tested at contraction intensities above 0 2 Nm were selected for a calculation of the average spindle frequency as a function of torque over a decent range in order to avoid the bias toward steep slopes from the unit tested exclusively at weak contractions The average discharge frequency was calculated from the equations fitted to the experimental data between 0 01 Nm and 1 0 Nm The filled circles in Fig 9 represent primaries and the triangles represent secondaries The points to the extreme left give the average resting discharge For the two curves the slopes were 32 8 ips/Nm and 22 8 ips/Nm respectively which figures fall reasonably well within the central regions of the regression coefficients of the single unit samples as shown in Fig 4 particularly if the values above 100 ips/Nm are neglected as there was some justification for The difference between the two curves in Fig 9 does not seem worth stressing Rather one would expect the secondaries to have generally higher frequencies all over because of sampling bias as the proportion of spontaneously active secondaries actually was higher than that of the primaries in he sample (*cf* Vallbo 1973 b)

*Effect of muscle length* 10 units were studied at 2 or 3 different joint angles with the purpose to assess to what extent the spindle frequency was dependent upon muscle length during lasting isometric contractions In all 13 pairs of observations were available for which the correlation coefficients were statistically significant When the difference in metacarpo-phalangeal joint angle was 10° (7 pairs) no systematic difference could be demonstrated in the pindle frequency as a function of

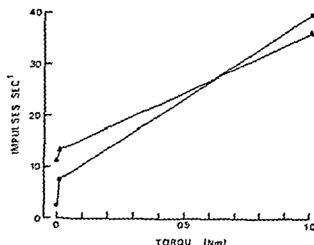



*Fig 9* Average discharge frequency of the spindle primary endings filled circles and of the secondary endings filled triangles as a function of the torque due to contraction The points to the extreme left represent the mean spontaneous discharge frequency whereas the other points and the lines between them indicate the mean impulse frequency during isometric voluntary contractions as calculated from linear equations fitted to the experimental data from the individual units. The diagrams are based upon data from 11 primaries and 5 secondaries suited for the analysis The lines represent the following two equations for the primaries and the secondaries respectively Y = 7 1 + 37 8 X Y = 13 2 + 22 8 X.

torque However for a difference of 20° the regression coefficient was higher when the muscle was longer for all the 4 primaries studied *i e* the frequency increased more steeply with torque when the muscle was longer whereas the regression coefficients were lower for the two secondaries studied when the muscle was longer It seems difficult to judge from this small sample whether the observed effects are occasional or indicate basic principles However the data presented on this point

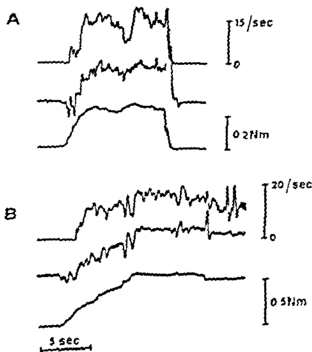



*Fig 10* Records to demonstrate the dependency of spindle discharge frequency of the torque as well as rate of the change of the torque due to isometric voluntary contractions The upper trace is an analogue representation of the discharge frequency from a spindle primary ending in the long finger flexor muscles produced by a filter circuit with a decay time constant of 0 11 s Lower trace shows the torque due to isometric voluntary flexion of the index Middle trace is a composite signal (S) of the analogue torque signal (T) and the negative time derivative of this signal scaled to match the upper signal The relative weight of these two summated signals are given by the following expressions for A and B respectively A S ∝ T —0 34 dT/dt B S ∝ T—0 43 dT/dt.

indicate that very pronounced effects of small changes of the joint angle did not occur.

*Spindle responses to changing torques.* Considering the scatter of the data in non-sequential displays as in Fig. 2, it seemed reasonable to analyse whether frequency was related not only to contraction intensity but also to other factors. It was found that some spindle primary endings obviously responded to the internal length changes in the muscle during isometric contractions. Examples are shown in Fig. 10. The top record is an analogue signal of the spindle frequency and the bottom one represents the torque during isometric voluntary contractions. The middle trace is a composite signal of the analogue torque signal and the negative time derivative of this signal, i.e. the torque signal and a signal of the rate of fall of the torque were added with appropriate scaling factors in order to simulate the spindle frequency. It may be seen that there was a striking agreement between the upper trace and the middle trace on many points, indicating that the spindle frequency was closely related to the skeletomotor output intensity as well as to the rate of change of the spindle length. An important physiological implication of the findings shown in Fig. 10 is that the fusimotor outflow during the voluntary contractions was not only of the static type but also of the dynamic type, as the ending had a pronounced dynamic sensitivity during the contraction while the fusimotor outflow was enhanced. It should be stressed, however, that dynamic responses of this type could not be clearly demonstrated in the majority of the primaries and the conditions under which they occurred were not analysed. The lack of agreement between spindle frequency and the predicted discharge on several points in Fig. 10 is not surprising considering that the afferent signal was recorded from one of a large number of sensory elements in a motor system of high complexity. The activity of the particular motor units which were most effective in unloading the spindle might not be all that well correlated to the total output of the muscle system as measured by the torque. Very striking changes of the spindle frequency without any corresponding changes of the torque are seen to the right in Fig. 10 B.

## Discussion

The main finding of the present study was that there was a close relation between impulse frequency in single spindle afferents and the strength of isometric contractions. This was seen most clearly in sequential displays showing how the spindle frequency continuously varied with the torque. For the further analysis the relation between torque and spindle frequency was described with linear equations. Obviously this implies an approximation as there was a considerable scatter of the data about the line and the study covered a limited number of torque levels for many units. Further, it was actually shown that there was a deviation from linearity in the low range of contraction intensities in that the frequency rose more steeply than it did at stronger contractions. The deviation suggests that a negatively accelerating curve might provide a better description over the full range of contraction intensities. However, adequate data were not available to allow a further analysis along these

lines and a linear relation seems at the present as the most reasonable description above the low torque range

The close relation between spindle frequency and torque due to active contraction suggests a similar relation between the intensity of the skeletomotor outflow and the fusimotor outflow from the spinal cord This statement in turn involves the assumption that there is a close relation between the two variables measured and the two motor outflows respectively The torque as determined in the present study seems an acceptable measure of the skeletomotor output intensity as the muscle length were not allowed to change and the contraction intensities were changing but slowly (Lippold 1952 Bigland and Lippold 1954 Coggshall and Bekey 1970 Stephens and Taylor 1972) It has been shown in animal experiments that the frequency of spindle afferent discharge is closely related to the impulse frequency in fusimotor neurones at constant muscle lengths This has been demonstrated on stimulation of single fusimotor fibres but it seems reasonable to assume that it is valid also when the system is active under natural conditions The relation is approximately a linear one except at very high fusimotor intensity (Matthews 1962 Andersson Lennerstrand and Thoden 1968 Lewis and Proske 1972)

Thus the data presented emphasize the close correspondence between the skeletomotor and the fusimotor outputs which now have been demonstrated in spatial temporal and intensive respects (Vallbo 1970 b 1971) This is not to claim that there is a rigid relation between the two motor outputs and that the relation cannot be altered from one type of motor act to the other However, it seems that the present findings define a basic principle with regard to the properties of the motor output under natural conditions and provide an essential requisite for more detailed studies of spindle responses in various types of motor activities including active movements.

There was often a considerable scatter of the data points from the individual unit This does not necessarily indicate a variation in the ratio between skeletomotor and fusimotor outflows but it may be accounted for by at least two other factors If there is a very close correspondence in spatial respect between the skeletomotor and the fusimotor outflows (*cf* Vallbo 1970 b) the discharge from a particular spindle would be closely related to the amount of activity in a particular muscle or group of motor units whereas the same torque output from the complex motor system studied may be produced by contractions of various spatial characteristics In addition it was actually shown that the spindle frequency may be dependent upon the rate of change of the torque

An increase of afferent discharge from spindle primaries at constant muscle length may be accounted for by either static and/or dynamic fusimotor activity (Matthews 1962 Crowe and Matthews 1964 Brown Crowe and Matthews 1965) In the present study several findings indicated that the static fusimotor outflow was prominent the very irregular discharge of the primaries (Bessou Laporte and Pagès 1968 Matthews and Stein 1969) and the fact that the discharge from the secondaries increased as well (Appelberg Bessou and Laporte 1966) The observed changes in impulse frequency with torque was similar for primaries and secondaries which might

suggest at first sight that the fusimotor outflow was largely limited to the static type However this is not a justified conclusion as there is a pronounced occlusion between static and dynamic fusimotor effects on the discharge of the primary endings at constant muscle length (Andersson *et al* 1968 Lennerstrand 1968 b) Although the evidence for a strong static fusimotor activation was compelling it seems important to stress that convincing evidence of dynamic fusimotor activation was also seen The fact that this was apparent only in a minority of the units may be accounted for by several factors The test contractions were not suited to reveal dynamic effects as the muscles were not allowed to shorten The dynamic fusimotor outflow may occur largely under particular conditions which were attained occasionally and by chance in the present study It seems important to point out however that the dynamic fusimotor system may participate in voluntary contractions and the present data are perfectly consistent with the dynamic fusimotor system being strongly and regularly activated in these types of contractions

The close relation found between the spindle afferent discharge and the intensity of the skeletomotor output is by itself consistent with the follow up length servo hypothesis (Merton 1951 1953 Hammond Merton and Sutton 1956) but it should be stressed that the present findings do not invalidate the evidence produced in earlier studies against this hypothesis for voluntary contractions (Vallbo 1971 1973 a)

Static fusimotor activity increases not only the spindle frequency at any muscle length but also the position sensitivity *i e* the slope of the steady state frequency versus muscle length even though this might not be true for the individual fusimotor unit (Jansen and Matthews 1962 b Crowe and Matthews 1964 a Lennerstrand and Thoden 1968 Schafer and Henatsch 1968 Brown Lawrence and Matthews 1969 Fromm and Haase 1970) Thus the present findings imply that a given change in muscle length would cause a larger amplitude modulation of the composite signal from the spindles the stronger the voluntary contraction On the assumption that the descending activity from the supraspinal structures onto the skeletomotor neurones also increases with the contraction strength (Evarts 1968 1969 Humphrey Schmidt and Thompson 1970) it follows that the relative role of the excitatory drive from these two principal sources onto the skeletomotor neurones may be retained unchanged as the contraction intensity varies Hence the extent to which the design of the skeletomotor output depends upon the appropriate peripheral events may be maintained as the sensitivity of the spindles increases with the fusimotor output It seems that a proportional skeletomotor and fusimotor outflow may provide a simple explanation of the finding that the effect of mechanical interference with a voluntary finger movement of constant velocity is similar in weak and strong contractions with regard to the proportional change in contraction intensity which finding has led to the postulation of a gain control in the loop from the muscle spindles to the central nervous system and back to the muscle (Marsden Merton and Morton 1972 a b)

Spindle frequency increased with torque more steeply in weak contractions than in strong contractions This probably reflects a similar difference in the relation

between fusimotor activation and torque in the two ranges The alternative interpretation i e that it was an effect of unloading seems unlikely as the compliance of the passive elements of the muscle decreases with force *The steep rise of fusimotor* outflow in weak contractions suggests that the functional state of the spindle is promptly adjusted as soon as a contraction starts to attain a level where it is well prepared to respond to muscle length changes *i e* the discharge is raised to a frequency which allows modulations in both directions

It has been shown that practically all the primary afferents from a muscle converge upon the individual skeleto motoneurone (Mendell and Henneman 1971) It seems therefore of some interest to consider the composite signal from all the spindles in a muscle In the long finger flexor muscles there are 682 spindles (Voss 1959) Under resting conditions the discharge from the 170 primary endings related to one of the four fingers would be 637 ips when the muscles are at their intermediate lengths as *in the present study (wrist joint dorsiflexed by 15° metacarpo-phalangeal joint 150* and interphalangeal joints at 180°) (Vallbo 1973 b) An isometric flexion of one finger would be associated with an abrupt increase from 637 ips up to at least 1 467 ips The last figure is based upon the earlier finding that at least 80 % of the spindles are activated even in weak voluntary contractions (Vallbo 1970 b) and the present findings concerning the average frequency from primary endings at a torque of 0 01 Nm (Fig 9 of the present paper) This is a very weak contraction approximately what is required to hold the finger against gravity As the contraction progresses further the discharge would reach about 5590 ips and 23 500 ips respectively at 25 % and 100 % of the maximal strength which is in the order of magnitude of 4 0 Nm for the index (Vallbo 1970 a) These values are 10 to 40 times the resting *discharge respectively The discharge at maximal contraction intensity* was calculated on the assumption that all the spindles were activated and not only 80 % of them Although there is a considerable uncertainty in these figures the order of magnitude is probably correct The high discharge during contraction strongly contrasts against the activity from the relaxed muscles

Considering on the other hand the maximal frequency from the individual spindle an extrapolation indicates that this would be 138 ips during a maximal contraction at intermediate muscle length This frequency is in the same order of magnitude as the frequency from cat hind limb muscle spindles during maximal activation of single fusimotor units and it is higher than the frequency from extensor spindles in decerebrate cats at maximal muscle length (Jansen and Matthews 1967 a b Lennerstrand and Thoden 1968 Lewis and Proske 1972) Only occasionally considerably higher frequencies have been observed at constant muscle length in animal experiments (Eldred Granit and Merton 1953) From this frequency range of around 150 ips considerable modulations are allowed in either direction during movements without saturation *of the spindle*

It has been suggested that an important function of the muscle spindles and the fusimotor system is to compensate for changes in load with regard to muscle length so that for instance the muscle length would be held approximately constant when

the load is increased (Merton 1951 Matthews 1964) This idea has in common with the follow up length servo hypothesis the basic requisite that the reflex stiffness accounted for by the stretch reflex loop is high in the steady state On the basis of data from the present study and an earlier investigation (Vallbo 1973 b) this point may be further elucidated for voluntary contractions in man if two assumptions are adopted Firstly that the central effects of the spindle and the tendon organ afferent discharge are the same during isometric contractions as studied in the present investigation and when the system is asked to resist varying external forces Second that the afferent input considered in the present study is representative of the total spindle input to the finger flexor motoneurone pool homonymous as well as heteronymous (Clough Kernell and Phillips 1968) An increase of the spindle afferent discharge induced by muscle stretch would give rise to an opposing skeletomotor contraction through the spinal reflex loop and possibly through more complex connections The amount of torque increase of the contracting muscles which may be produced by a given change in spindle afferent activity in face of the simultaneous increase of inhibition from other sources Golgi tendon organs and possibly spindle secondary endings is set by the average slope of 32 8 ips/Nm as found in the present study Hence an average increase of the spindle discharge by 1 0 ips would give rise to an increase of the torque by 0 03 Nm This is approximately equivalent to 30 g weight applied at the tip of the finger Obviously the spindle afferent activity would have such a strong effect only if the excitatory drive onto the skeletomotor neurones originated exclusively from the spindle afferents and the effect of any descending activity from supraspinal structures onto the motoneurones would be negligible This seems rather unlikely (Koeze Phillips and Sheridan 1968 Vallbo 1971) and hence the figure is probably too high However the important point for the present reasoning is that the value is a rigid upper limit on the assumptions specified In order to assess to what extent the spindle input is strong enough to account for holding the muscle at nearly constant length when the load varies the position sensitivity of the spindle must be known Data are not available for spindles in contracting human muscles but the order of magnitude may be inferred on the basis of data from animal experiments and from relaxed human muscles In cat hind limb muscle spindles it seems that an average increase of the position sensitivity by a factor of 5 from the de efferented spindle position sensitivity is a maximum which may be attained at strong fusimotor activation (Jansen and Matthews 1962 Lennerstrand 1968 b Lennerstrand and Thoden 1968 Brown Lawrence and Matthews 1969) Assuming that this factor is an upper limit also for human finger flexor muscle spindles it follows that the average position sensitivity may increase from 0 18 ips/degree at the metacarpo phalangeal joint angle in the relaxed muscle (Vallbo 1973 b) to 0 90 ips/degree in strong contractions The position sensitivity may be considerably lower in contractions of moderate intensity

On the basis of these considerations it follows that the proprioceptive reflex effects from the muscle would account for a stiffness of 0 027 Nm/degree at the metacarpo-phalangeal joint of a single finger Obviously the contracting muscle would not at

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# Cerebral Blood Flow and Cerebral Metabolic Rate for Oxygen in Rats with Porta-Caval Anastomosis

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## Abstract

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Regional cerebral blood flow and cerebral arteriovenous differences in oxygen content were studied in rats provided with a porta-caval shunt The arteriovenous oxygen differences decreased while cerebral blood flow was unchanged It is concluded that rats with a porta-caval anastomosis have a reduction in cerebral metabolic rate for oxygen possibly due to reduced functional demands

Patients with hepatic encephalopathy have a reduced cerebral metabolic rate for glucose and oxygen and a parallelity seems to exist between this reduction and the severity of the neuropsychiatric symptoms (Fazekas *et al* 1956 Erbsloh *et al* 1958 Posner and Plum 1960) Since the detoxification of ammonia in the brain requires energy it has been assumed that the reduction in cerebral metabolic rate for oxygen can be explained as a primary interference of ammonia with the energy metabolism of the brain Some support for this view is given by Schenker *et al* (1967) and Hindfelt (1972) who observed that ammonia intoxication in rats is accompanied by slight decreases in the phosphocreatine and ATP contents in brain structures However hyperammonemia in rats provided with a surgically constructed porta-caval shunt is not associated with signs of a disturbed energy balance in the brain (Holmin and Siesjo 1973 a) Furthermore when shunted rats are exposed to arterial hypoxia (Holmin and Siesjo 1973 b) or to arterial hypotension (Holmin *et al* 1973) the metabolic changes in the brain are similar to or even less pronounced than those observed in the corresponding control animals

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Shunted rats exposed to hypotension had higher oxygen tensions in cerebral venous blood than nonshunted control rats (Holmin *et al* 1973) This finding either indicates a decreased consumption of oxygen in the brain and/or an increased cerebral blood flow

The objective of the present study was to determine regional cerebral blood flow and cerebral metabolic rate for oxygen in rats provided with a porta caval shunt Regional cerebral blood flow was measured with a modified $^{14}C$ antipyrine method (Reivich *et al* 1969 Eklof and Siesjo 1973) Determination of oxygen content in arterial blood and in cerebral venous blood allowed calculations of cerebral metabolic rate for oxygen

## Methods

A *Operative and sampling techniques* Male Wistar rats (260—375 g) were used The rats were provided with an end to side porta caval anastomosis using a technique described in detail in an earlier communication (Holmin and Siesjo 1973 a) The rats were then allowed to wake up and had free access to water and rat pellets (San Bolagen Malmo) One week after the establishment of the shunt the rats were reanesthetized with divinyl ether immobilized with tubocurarine chloride tracheotomized and ventilated artificially with 70 % $N_2O$ and 30 % $O_2$ using Starling type respirators The body temperature measured in the rectum was kept close to 37 C The animals were heparinized The femoral arteries as well as the femoral veins were cannulated One arterial catheter was used for continuous blood pressure recording and the other for anaerobic sampling of blood One femoral vein was used for infusion of $^{14}C$ antipyrine and the other for injection of saturated potassium chloride at the end of the experiment. A skin incision was made over the skull bone and a hole was burred with a fine drill over the superior sagittal sinus for later sampling of blood in a glass capillary Nonshunted rats were used as controls

B *Experimental design* The operative procedures were followed by a steady state period of about 60 min During that period at least 3 arterial samples were taken for measurements of pH $P_{CO}$ and $P_O$ The respirators were set so as to give a normal arterial $P_{CO_2}$ (35—40 mm Hg) Every animal had an arterial $P_{O_2}$ higher than 120 mm Hg The last arterial sample taken 5 min before the infusion of $^{14}C$ antipyrine was also used for measurements of oxygen content. At the same time a sample was taken from the superior sagittal sinus for measurement of oxygen content The $^{14}C$ antipyrine was given i v during 60 s by means of an infusion machine (administered activity 50 $\mu$Ci/kg of b wt in 1 ml of an isotonic NaCl solution) During that period arterial blood was sampled every 5th s in 50 $\mu$l glass capillaries for measurements of $^{14}C$ antipyrine activity Immediately after the last arterial sample had been taken one ml of saturated potassium chloride was injected as rapidly as possible and the rat was decapitated within 2—3 s The head was frozen in liquid nitrogen and stored at −80 C until dissected and analysed

C *Analytical techniques* Arterial $P_O$ $P_{CO}$, pH and hemoglobin concentration were measured and the Base excess value was calculated as previously described (Holmin and Siesjo 1973 a) The oxygen content in arterial and in cerebral venous blood was determined by using an oxygen electrode (Fschweiler) according to Fabel and Lubbers (1964) The coefficient of variation for the determination was less than 2 per cent (Hagerdal Lewis and Pontén in preparation)

The $^{14}C$-activity in blood and brain tissue was determined according to Eklof and Siesjo (1973) Aliquots of 40 $\mu$g of blood were added to scintillation vials containing Soluene® and isopropanol and hydrogen peroxide was added in order to diminish colour quenching Brain tissue samples (about 50 mg) were dissected and weighed in a box at −10 C The samples were dissolved in Soluene® Instagel® was used as scintillation system for both blood and brain tissue and the $^{14}C$ activity was measured in a liquid scintillation counter Samples from the frozen brain were taken from 16 areas brainstem cerebellar cortex frontal cortex parietal border zone cortex parietal non border zone cortex occipital cortex temporal basal cortex and deep hemispheral structures The border zone cortex included the border zone between the distribution territories of the anterior and the middle cerebral arteries while the non border zone cortex was taken from the distribution territory of the middle cerebral artery

TABLE I Body weight body temperature mean arterial blood pressure (MABP) hemoglobin concentration (Hb) arterial oxygen tension ($P_{O_2}$) and arterial acid base parameters of control rats and of rats with a porta caval anastomosis Means ± S E n = number of animals

| Exp group | Weight g | Temp C | MABP mm Hg | Hb g | $P_{O_2}$ mm Hg | pH | $P_{CO_2}$ mm Hg | BE mequiv /l |
|---|---|---|---|---|---|---|---|---|
| Controls | 313 | 36 6 | 150 | 17 5 | 146 | 7 385 | 36 0 | −2 5 |
| (n = 9) | ±15 | ±0 1 | ±2 | ±0 4 | ±4 | ±0 012 | ±0 6 | ±0 7 |
| P-c shunt | 306 | 37 0* | 130*** | 15 7* | 138 | 7 383 | 37 9* | −1 9 |
| (n = 11) | ±9 | ±0 1 | ±2 | ±0 4 | ±4 | ±0 005 | ±0 4 | ±0 4 |

*Calculations* For calculations of the regional cerebral blood flow (rCBF) the equation given by Kety (1960) was used

$$C_i(T) = \lambda K_i \int_0^T C_a\, e^{-K_i(T-t)}\, dt$$

where $C_i$ (T) is the brain tissue concentration of $^{14}C$ antipyrine at time T In the present study T was 61 s $\lambda$ is the blood brain partition coefficient for $^{14}C$ antipyrine $K_i$ is the rate of blood flow per unit weight of brain tissue multiplied by the reciprocal of the partition coefficient for that tissue and $C_a$ is the concentration of $^{14}C$ antipyrine in arterial blood Since the arterial catheter used was very short (15—20 mm) no correction was made for the smearing factor of the sampling catheter A program was written for the Varian computor 620/i to calculate by means of the trapezoid rule the value of $C_i$ (T) for various values of K chosen according to a simple algorithm to reach the proper K

*Statistical analyses* Wilcoxon s rank sum test was used ($p < 0\ 05$ = * $p < 0\ 02$ = ** and $p < 0\ 01$ = *** )

## Results

Table I shows general physiological parameters as well as acid base values in non shunted control rats and in rats provided with a porta caval anastomosis The non shunted control group had a slightly higher body temperature and a somewhat lower arterial $P_{CO_2}$ than the shunted rats

Table II shows the CBF values in the cerebellum brainstem and different regions of the two hemispheres The shunted rats had a slightly higher rCBF value in the left non border zone parietal cortex but in all other structures the rCBF values were similar in shunted rats and in controls This difference may have been fortuitous and the data demonstrate that the porta caval anastomosis did not alter rCBF There were relatively small differences in flow between different brain structures However the rCBF values in the brainstem cerebellum and deep hemispheral parts were lower than in other parts of the brain The mean rCBF values for brainstem and cere bellum were lower in the shunted group but the differences obtained were not sta tistically significant If the values for brainstem and cerebellum are compared to the calculated mean cortical blood flow (see below) there were highly significant dif ferences in the shunted group ($p < 0\ 01$) but no significant differences in the con trol group This difference in flow is of interest in the light of previously reported regional differences in metabolites during ammonia intoxication and in port anastomosis (Schenker *et al* 1967 Hindfelt 1972 Holmin and Siesjo 1973

TABLE II Regional cerebral blood flow of control rats and of rats with porta caval anastomosis Means ± S E in ml/100 g × min n = number of rats

| Part of brain | | Controls (n = 9) | P c shunt (n = 11) |
|---|---|---|---|
| Brainstem | | 67 ± 2 | 61 ± 3 |
| Cerebellum | | 66 ± [illegible] | 62 ± 2 |
| Frontal cortex | right | 78 ± 2 | 79 ± 3 |
| | left | 77 ± 3 | 83 ± 4 |
| Parietal cortex border zone | right | 71 ± 4 | 73 ± 3 |
| | left | 70 ± 3 | 74 ± 2 |
| Parietal cortex non border zone | right | 73 ± 3 | 78 ± 4 |
| | left | 71 ± 3 | 80 ± 4* |
| Temporal basal cortex | right | 70 ± 3 | 69 ± 4 |
| | left | 70 ± 3 | 70 ± 3 |
| Occipital cortex | right | 7[illegible] ± 3 | 72 ± 3 |
| | left | 71 ± 2 | 78 ± 3 |
| Deep hemispheral structure | right | 67 ± 2 | [illegible] ± 2 |
| | left | [illegible]7 ± 2 | 63 ± 2 |

TABLE III Contents of oxygen (TO) in arterial and cerebral venous blood of control rats and of rats with a porta caval shunt AVD = arteriovenous difference n = number of rats Means ± S E in [illegible]

| Exp group | TO | | |
|---|---|---|---|
| | arterial | venous | AVD |
| Controls (n = 9) | 22 7 ±0 7 | 14 1 ±0 6 | 8 6 ±0 6 |
| P c shunt (n = 11) | 20 4** ±0 5 | 13 8 ±0 [illegible] | 6 6 ±0 4 |

Table III shows the oxygen contents in arterial and in cerebral venous blood. The shunted rats had a significantly reduced arterial oxygen content corresponding to the somewhat lower blood hemoglobin concentration (see Table I). Since the venous oxygen contents were similar in the two groups the a v oxygen difference was significantly reduced in the shunted group. The cerebral venous blood was taken from the superior sagittal sinus draining the frontal and parietal cortex of the brain. A mean cortical blood flow (MCBF) was derived from the six cortical regions analysed and this MCBF was used for calculation of cerebral metabolic rate for oxygen. Table IV shows the mean cortical CBF values and the calculated cerebral metabolic rates for oxygen. The shunted rats had an unchanged mean cortical blood flow but a reduced cerebral metabolic rate for oxygen.

TABLE IV Mean cortical blood flow (MCBF see text) and calculated cerebral metabolic rate ($CMR_O$) for oxygen in the brains of control rats and shunted rats Means ± S E n = number of animals

| Exp group | MCBF ml/100 g × min | $CMR_O$ ml/100 g × min |
|---|---|---|
| Controls (n = 9) | 73 6 ±2 3 | 6 24 ±0 30 |
| P-c shunt (n = 11) | 76 6 ±3 1 | 5 02** ±0 32 |

TABLE V Regional cerebral blood flow (rCBF) of the rat according to different authors

| Author | Method | Anesthesia | $Pa_{CO_2}$ mm Hg | rCBF ml/100 g × min cortex | cerebellum |
|---|---|---|---|---|---|
| Kennedy 1971 | ¹ C-antipyrine autoradiogram | awake | 39 | 81 | 53 |
| Haining *et al* 1970 | hydrogen clearance | awake | — | 77 | 65 |
| Pannier and Leusen 1973 | microspheres | nembutal | 35 | 75 | — |
| Goldman and Sapirstein 1973 | C-antipyrine direct counting | awake | 40 | 103 | 92 |
| Present study | C-antipyrine direct counting | 70 % N O | 36 | 74 | 66 |

## Discussion

Patients with hepatic encephalopathy show a reduction in the cerebral metabolic rate for oxygen ($CMR_O$) that is proportional to the severity of the cerebral symptoms In overt coma the $CMR_O$ is reduced to about 50 % of normal (Wechsler *et al* 1954 Posner and Plum 1960) but a significant reduction in metabolic rate is also observed in patients that are lethargic and mildly confused (Posner and Plum 1960) Since the degree of encephalopathy correlates rather closely with the ammonia concentrations in blood (McDermott *et al* 1954) and since acute ammonia intoxication in experimental animals leads to coma it has been assumed that both the cerebral symptoms and the reduction in $CMR_{O_2}$ reflect an influence of ammonia on the brain The mechanisms that detoxify ammonia in the brain are energy requiring in that they either encroach upon energy production from breakdown of carbohydrate substrates or consume ATP directly It has therefore seemed natural to assume that ammonia intoxication induces cerebral symptoms and a decrease in $CMR_{O_2}$ by reducing production of energy in the tissue There are though no unequivocal evidence for this Thus although Schenker *et al* (1967) reported decreases in the brainstem concentrations of ATP and phosphocreatine in acute ammonia intoxication (*cf* also Hindfelt 1972) other results are somewhat contradictory in that they gave no clear evidence of a fall in ATP in the absence of convulsions (Hindfelt and Siesjo 1971)

We have recently analysed organic phosphates carbohydrate substrates and amino acids in the brains of rats with sustained hyperammonemia due to porta caval anastomosis (Holmin and Siesjo 1973 a) In two other series shunted rats were stressed with either arterial hypoxia (Holmin and Siesjo 1973 b) or arterial hypo tension (Holmin et al 1973) In none of these studies were there signs of an im balance between production and utilization of energy in the tissue and the results therefore leave no support to the hypothesis that ammonia intoxication critically decreases energy production in the brain In fact the shunted rats withstood a reduc tion in arterial blood pressure to 30 mm Hg better than the controls (Holmin et al 1973) It was observed in the latter study that the shunted rats had a higher cerebral venous $P_{O_2}$ than the controls bled to the same mean arterial blood pressure The present experiments were performed to establish if the higher venous $P_{O_2}$ in the shunted rats is due to an increase in cerebral blood flow or to a reduction in $CMR_O$ Before discussing the results it seems appropriate to consider the methods used to measure rCBF and $CMR_O$

*Measurements of rCBF* The $^{14}C$ antipyrine method (Reivich et al 1969) is based on principles established by Kety (1951) and was originally used for autoradio graphic quantification of the regional cerebral blood flow In the modification de scribed by Eklof and Siesjo (1972) samples were dissected from different areas of the brain and the $^{14}C$ activity was determined by liquid scintillation counting The validity of the method depends on instantaneous diffusion of antipyrine between blood and tissue Since antipyrine may not diffuse quickly enough to meet the re quirements of the method (Crone 1965) the values obtained may be in error How ever the present rCBF values agree rather closely both with previous antipyrine data and with results obtained in the rat using other methods (Table V) Furthermore since the values for shunted rats and controls were similar they should be valid for comparisons even if the abolute values are somewhat uncertain

*Measurements of $CMR_{O_2}$* The a v oxygen differences measured between arterial blood and blood from the superior sagittal sinus in all probability reflect $CMR_{O_2}$ in cortical tissue from the frontal and parietal regions However, since the tissue regions drained by the superior sinus are not accurately defined the $CMR_{O_2}$ obtained can only be assumed to represent a cortical metabolic rate There are reasons though to assume that this uncertainty does not affect the conclusions drawn Firstly the rCBF values obtained for several cortical regions were very similar Secondly, dif ferences in $CMR_{O_2}$ between shunted rats and controls are obtained even if cal culated from rCBF values for subcortical structures

*Significance of rCBF values* Patients with advanced encephalopathy show a re duction in CBF Wechsler et al 1954 Posner and Plum 1960) In patients with lethargy and mild confusion Posner and Plum (1960) reported 20 per cent reduc tion in CBF and a 15 per cent reduction in $CMR_{O_2}$ James et al (1971) found a 30 per cent decrease in CBF and a 20 per cent decrease in $CMR_O$ in dogs studied one hour after porta caval shunting However in view of the fact that the CBF varies by about 3 per cent for each mm Hg change in $Pa_{CO}$ (Kety and Schmidt

1948) the observed changes in CBF in moderate encephalopathy are not necessarily due to the encephalopathy The present results of an unchanged CBF in animals that show no abnormal behaviour are therefore not inconsistent with previous results in man (*cf* also Maiolo *et al* 1971)

*Significance of* $CMR_{O_2}$ *values* The results reported by Posner and Plum (1960) demonstrate that there is a significant reduction in $CMR_{O_2}$, even in the absence of coma or somnolence In patients such subtle changes as lethargy and mild confusion can easily be assessed In the rat an apparent normal behaviour cannot be taken as evidence of a normal cerebral function and the reduction in $CMR_{O_2}$ observed in the present shunted animals is therefore consistent with a moderate degree of encephalopathy

The present results allow a more firm conclusion with regard to the influence of hyperammonemia on the brain in rats with a porta caval anastomosis Shunted rats have a sustained hyperammonemia (Lee and Fisher 1961 Cavanagh and Kyu 1971 Holmin and Siesjö 1973 a) show grossly increased glutamine contents in the brain (Williams *et al* 1972 Holmin and Siesjo 1973 b) and develop morphological changes affecting the astrocytes (Cavanagh and Kyu 1971) It has also been reported that rats with a porta-caval shunt develop muscular hypertonicity and electro encephalographic changes (Degos *et al* 1969) These changes as well as the de crease in $CMR_{O_2}$ demonstrated in the present material indicate that the shunting procedure is accompanied both by signs of ammonia detoxification and by a toxic influence on the brain that is possibly due to ammonia Since these signs are present in spite of normal concentrations of adenine nucleotides in supra as well as infra tentorial structures we conclude that the decrease in $CMR_{O_2}$ is unrelated to a previ ous derangement of the balance between production and utilization of energy in the brain It must remain for future experiments to establish how porta caval shunts and hyperammonemia influence cerebral functions

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# Effects on Cerebral Energy State of Arterial Hypotension in Rats with Porta Caval Anastomosis

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## Abstract

HOLMIN T H JOHANNSSON and B K SIESJO *Effects on cerebral energy state of arterial hypotension in rats with porta caval anastomosis* Acta physiol scand 1974 *90* 345—357

The influence of arterial hypotension upon the energy state of the brain was studied in rats with porta-caval anastomosis by bleeding the animals to mean arterial blood pressures of 45 and 30 mm Hg respectively Reduction of blood pressure to 45 mm Hg in shunted rats gave no or only very moderate accumulation of lactate and there were no changes in the contents of ATP ADP and AMP in frontal lobe or brainstem At a mean blood pressure of 30 mm Hg shunted animals and controls showed similar values for glycolytic and citric acid cycle intermediates amino acids and adenine nucleotides in frontal lobe parietal cortex and brainstem It is concluded that the shunting procedure and the ensuing hyperammonemia does not adversely affect the sensitivity of the cerebral energy state to arterial hypotension

Patients with cirrhosis of the liver and porta-caval anastomosis often develop hepatic encephalopathy (Read *et al* 1961 see also Martini 1971) These patients show a reduction in cerebral metabolic rate for oxygen (Fazekas *et al* 1956 Posner and Plum 1960 Maiolo *et al* 1971) which is proportional to the severity of the encephalopathy Reversible episodes of encephalopathy correlate rather closely with increases in blood ammonia levels (McDermott *et al* 1954) and since experimental hyperammonemia leads to coma (Schenker and Mendelson 1964 Schenker *et al* 1967) it is often assumed that at least part of the symptomatology of hepatic encephalopathy is due to ammonia intoxication

Since the detoxification of ammonia in the brain either interferes with energy production from carbohydrate substrates or consumes ATP directly (see Schenker *et al* 1967 Hindfelt and Siesjo 1971) it has been assumed that hyperammonemic coma is secondary to depletion of energy in the tissue This assumption receives some support from results showing slight reductions in the phosphocreatine and ATP concentrations of brainstem structures in experimental hyperammonemia (Schenker *et al* 1967 see also Hindfelt 1972) However when rats are made chronically hyper

ammonemic by means of a porta-caval anastomosis the ATP, ADP and AMP contents of the frontal lobe, brainstem and cerebellum remain unaltered (Holmin and Siesjö 1973 a). Furthermore, when such rats are exposed to arterial hypoxia they develop changes in the cerebral metabolic state that are very similar to those seen in hypoxic control animals (Holmin and Siesjö 1973 b). These results demonstrate that the detoxification of ammonia in chronic hyperammonemia does not necessarily disrupt the cerebral energy state, and they indicate that hyperammonemic coma could have other causes than a depletion of the energy stores (cf. Hindfelt and Siesjö 1971).

The present experiments were undertaken to study the influence of arterial hypotension upon the cerebral metabolic state in rats with porta-caval anastomosis. In non-shunted rats the energy state of the tissue is affected first when the mean arterial pressure is decreased to 30—45 mm Hg (Reulen *et al.* 1968, Kaasik *et al.* 1969, Siesjö and Zwetnow 1969, Eklöf *et al.* 1972). In one part of the present study the blood pressure of shunted rats was acutely decreased to 45 mm Hg by means of controlled bleeding, and the results were compared to those obtained in shunted rats with a normal blood pressure. In another part, the blood pressure was decreased to 30 mm Hg and a comparison was made to non-shunted rats bled to the same mean arterial pressure. In all animals phosphocreatine and adenine nucleotides were measured together with selected carbohydrate substrates and amino acids.

## Methods

*Operative and sampling techniques.* Male Wistar rats, weighing 300—360 g, were provided with an end-to-side porta-caval shunt, using a technique described in detail in an earlier communication (Holmin and Siesjö 1973 a). The animals recovered rapidly after the operation and did not show any abnormal behaviour. One week after the shunting procedure the animals were reanesthetized with diethyl ether, immobilized with tubocurarine chloride, tracheotomized and ventilated artificially with 70 % $N_2O$ and 30 % $O_2$, using Starling type respirators. The animals were heparinized. The body temperature was kept close to 37° C by means of a heating lamp. The femoral arteries were cannulated for continuous electromanometric blood pressure recording, for anaerobic sampling of blood, and for bleeding into a constant pressure reservoir. The distal part of the superior sagittal sinus was exposed for sampling of cerebral venous blood. At the end of the experiment a plastic funnel was fitted into a skin incision over the intact skull bone. For freezing the brain *in situ* liquid nitrogen was poured into the funnel during continuous ventilation (see Holmin and Siesjö 1972 a).

About 10 min after the preparation of the animal was completed, arterial and cerebral venous blood were sampled for measurements of pH, $P_{CO_2}$ and $P_{O_2}$. A second arterial sample was taken about 10 min later to make sure that a respiratory steady state was present ($P_{CO_2}$ constant to within 1.0 ...). The arterial $CO_2$ tension was set so as to give similar tissue $CO_2$ tensions in all groups (see below). The animals were then either bled to mean blood pressures of 45 and 30 mm Hg, respectively, or were maintained at normal blood pressure. In the bled animals the pressure was kept constant for 30 min. Fifteen as well as 30 min after the desired blood pressure level was attained samples were taken from the femoral artery and from the superior sagittal sinus for measurements of pH, $P_{CO_2}$ and $P_{O_2}$. The blood hemoglobin concentration was measured in the 30 min sample. The brain tissue was then frozen *in situ*, chiselled out with cold instruments and stored at −80° C until analysed.

*Analytical techniques.* The pH, $P_{CO_2}$ and $P_{O_2}$ were measured at 37° C with microelectrodes and the values were corrected for temperature. Hemoglobin was determined photometrically. The brains were dissected, weighed and extracted with methanol-HCl-$HClO_4$ at — ° C (Lowry and Passonneau 1972). The brain regions dissected were the frontal lobe (anterior to the middle cerebral artery), the parietal cortex with adjacent white matter, the cerebellum

TABLE I Mean arterial blood pressure (MABP) rectal temperature hemoglobin concentration arterial $P_{O_2}$ and arterial acid base parameters in shunted and control rats BE = base excess Means±S E n = number of animals

| Exp group | MABP mm Hg | Temp C | Hb g/100 ml | $P_{O_2}$ mm Hg | $P_{CO}$ mm Hg | pH | BE mequiv /l |
|---|---|---|---|---|---|---|---|
| P-c shunt (n = 7) | 125 ±4 | 36 8 ±0 1 | 13 5 ±0 5 | 146 ±3 | 37 7 ±0 4 | 7 35 ±0 01 | −4 1 ±0 8 |
| P-c shunt (n = 7) | 45 | 36 9 ±0 1 | 14 7 ±0 7 | 150 ±2 | 32 4 ±0 6 | 7 24* ±0 02 | −12 5*** ±1 1 |
| Controls (n = 9) | 30 | 37 1 ±0 2 | 10 8 ±0 7 | 160 ±8 | 28 4 ±1 7 | 7 04 ±0 02 | −22 4 ±0 8 |
| P-c shunt (n = 9) | 30 | 37 1 ±0 2 | 9 1 ±0 7 | 162 ±6 | 28 9 ±1 3 | 7 08 ±0 03 | −20 2 ±1 1 |

and the brainstem Since the parietal sample contained predominantly grey matter it will be referred to as parietal cortex The metabolites analysed included phosphocreatine ATP ADP AMP glucose glucose 6 phosphate (G6P) pyruvate lactate citrate α ketoglutarate (α KG) malate glutamate aspartate and ammonia All metabolites were measured with fluorometric enzymatic techniques (Lowry and Passonneau 1972) in a manner described previously (Folbergrova *et al* 1972 a and b)

*Calculations* The plasma bicarbonate concentration was calculated from pH and $P_{CO}$ using the solubility factor and pK given by Severinghaus (1965) with corrections for temperature and pH The blood base excess concentration was calculated according to Siggaard Andersen (1966) The tissue $CO_2$ tension was calculated from the arterial and cerebral venous $CO_2$ tensions as described by Ponten and Siesjo (1966) The relationship between the mean tissue CO tension thus calculated and the CSF $CO_2$ tension has been shown to be valid in hypotensive conditions (Eklof *et al* 1972) and in rats with a porta caval shunt (Holmin and Siesjo 1973 a) The energy charge of the adenine nucleotide system (ECP) was calculated from ATP ADP and AMP according to Atkinson (1968)

*Statistical analyses* Wilcoxon s rank sum test was used to calculate statistical differences ($p < 0\ 05$ = * $p < 0\ 02$ = ** and $p < 0\ 01$ = ***)

TABLE II Arterial and cerebral venous CO tensions arterio-venous $P_{CO}$ differences and calculated tissue CO tensions in shunted and control rats Means±S E in mm Hg n = number of animals AVD = arteriovenous difference

| Exp group | MABP | $P_{CO}$ arterial | venous | AVD | tissue |
|---|---|---|---|---|---|
| P c shunt (n = 7) | 125 ±4 | 37 7 ±0 4 | 50 4 ±0 8 | 12 6 ±1 0 | 45 1 ±0 4 |
| P-c shunt (n = 7) | 45 | 32 4 ±0 6 | 52 7 ±1 2 | 20 4 ±1 3 | 43 5 ±0 7 |
| Controls (n = 9) | 30 | 28 4 ±1 7 | 61 9 ±2 4 | 33 5 ±3 3 | 46 1 ±1 3 |
| P c shunt (n = 9) | 30 | 28 9 ±1 3 | 57 0 ±2 5 | 28 0 ±1 9 | 44 0 ±1 7 |

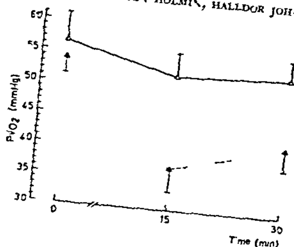



Fig 1 Oxygen tensions in cerebral venous blood of normotensive or hypotensive (45 mm Hg) rats supplied with a porta-caval shunt. Open triangles denote mean values for normotensive rats and filled triangles for hypotensive rats. Vertical bars denote ± S E (n = 7)

## Results

In designing the experiments attempts were made to avoid variations in body temperature arterial $P_O$ or tissue $CO_2$ tension. Table I shows that in the shunted group bled to a mean arterial blood pressure of 45 mm Hg body temperature blood hemoglobin concentration and arterial O tension were similar to the controls. The bled animals developed a nonrespiratory acidosis. The arterial $P_{CO}$ of the hypotensive animals was maintained somewhat lower than in the controls in order to keep an unchanged tissue CO tension (see below)

In the 30 mm Hg groups the body temperature hemoglobin concentration arterial $P_O$ and arterial acid base parameters were similar in shunted animals and

TABLE III The contents of ammonia ($NH_4$) glucose (GLC) glucose-6-phosphate G6P) lactate LAC) pyruvate (PYR α ketoglutarate (α KG) glutamate GLU and aspartate ASP in different parts of the brains of shunted normotensive or hypotensive rats Means ± S E in mmol/kg wet tissue n = number of animals

| Part of brain | Exp group | $NH_4$ | GLC | G6P | LAC | PYR | α KG | GLU | ASP |
|---|---|---|---|---|---|---|---|---|---|
| Frontal Lobe | Normotension n = 7 | 0.[illegible]1 ±0.0[illegible] | 2.8[illegible] ±0.12 | 0.099 ±0.009 | 2.01 ±0.0[illegible] | 0.103 ±0.003 | 0.116 ±0.009 | 10.98 ±0.19 | [illegible].8[illegible] ±0.08 |
| | 45 mm Hg n = [illegible] | 0.90 ±0.0[illegible] | 2.81 ±0.37 | 0.119 ±0.004 | 2.41 ±0.15 | 0.113 ±0.004 | 0.10[illegible] ±0.003 | 10.54 ±0.13 | [illegible] ±0.0[illegible] |
| Cerebellum | Normotension n [illegible] | 0.43 ±0.0[illegible] | 3.95 ±0.37 | 0.044 ±0.013 | 1.[illegible] ±0.08 | 0.08[illegible] ±0.00[illegible] | 0.0[illegible]4 ±0.010 | 9.90 0.13 | [illegible].2[illegible] ±0.0[illegible] |
| | 45 mm Hg n [illegible] | 0.[illegible] –0.03 | 2.4[illegible] ±0.4[illegible] | 0.068 ±0.013 | 2.07 ±0.11 | 0.081 ±0.003 | 0.0[illegible]6 ±0.006 | 10.00 ±0.29 | [illegible].29 ±0.0[illegible] |
| Brainstem | Normotension (n = [illegible] | 0.[illegible] ±0.03 | 2.67 ±0.23 | 0.030 ±0.010 | 1.64 ±0.10 | 0.0[illegible] ±0.004 | 0.03[illegible] ±0.00[illegible] | [illegible].87 ±0.[illegible] | 3.1 ±0.0[illegible] |
| | 45 mm Hg (n = [illegible] | 0.3[illegible] 0.0[illegible] | 2.4[illegible] ±0.[illegible]7 | 0.04[illegible] ±0.014 | 2.4[illegible]* ±0.47 | 0.0[illegible]3 ±0.008 | 0.038 ±0.004 | [illegible] 0.13 | 3.[illegible] ±0.1[illegible] |

TABLE IV Contents of phosphocreatine (PCr) ATP ADP and AMP in the frontal lobe and the brainstem of shunted normotensive or shunted hypotensive rats The calculated energy charge potential (ECP) and the sum (S) of adenine nucleotides are also given Means ± S E in mmol/kg wet tissue n = number of rats

| Part of brain | Exp group | PCr | ATP | ADP | AMP | ECP | S |
|---|---|---|---|---|---|---|---|
| Frontal lobe | Normotension (n = 7) | 4.53 ±0.03 | 3.12 ±0.01 | 0.260 ±0.006 | 0.038 ±0.001 | 0.951 ±0.001 | 3.42 ±0.02 |
| | 45 mm Hg (n = 7) | 4.30* ±0.07 | 3.20 ±0.02 | * 0.269 ±0.005 | 0.036 ±0.001 | 0.952 ±0.001 | 3.51 ±0.02 |
| Cerebellum | Normotension (n = 6) | 5.93 ±0.15 | 2.89 ±0.05 | 0.327 ±0.047 | 0.054 ±0.009 | 0.934 ±0.010 | 3.27 ±0.02 |
| | 45 mm Hg (n = 5) | 6.10 ±0.09 | 3.03 ±0.03 | 0.254 ±0.023 | 0.038 ±0.003 | 0.950 ±0.004 | 3.31 ±0.02 |

in controls In both groups there were a reduction in hemoglobin concentration and a marked nonrespiratory acidosis

Table II shows the arterial and cerebral venous $P_{CO_2}$ values the $P_{CO_2}$ differences and the calculated tissue $CO_2$ tensions The hypotensive states were associated with increased arteriovenous $P_{CO_2}$ differences However the arterial $CO_2$ tensions were adjusted sufficiently to give similar tissue $CO_2$ tensions in all groups

A *Reduction in blood pressure to 45 mm Hg* The material consisted of two shunted groups In one the mean blood pressure was reduced to 45 mm Hg for 30 min The other one the control group was a normotensive group maintained at normal arterial $P_{CO_2}$ Fig 1 shows that the venous $P_{O_2}$ was reduced both at 15 and 30 min ($p < 0.05$) when compared to the control group This reduction in venous $P_{O_2}$ and the increased arteriovenous $P_{CO_2}$ difference (see above) is consistent with a fall in cerebral blood flow

Table III shows the ammonia contents and the contents of the carbohydrate substrates and amino acids in the frontal lobe the cerebellum and the brainstem The mean values for ammonia content were somewhat higher in the hypotensive animals but the differences were not statistically significant ($p > 0.05$) Apart from an increase in lactate content in the brainstem there were no significant changes in the measured carbohydrate substrates and amino acids between the groups

Table IV illustrates the organic phosphates in the frontal lobe and the cerebellum (a sufficient number of analyses on brainstem was not obtained) In the frontal lobe the hypotensive group showed a slight fall in phosphocreatine The sum of the adenine nucleotides increased mainly due to a higher ATP content Since the calculated adenylate energy charge did not differ between the hypotensive and normotensive groups there were no tissue changes to suggest that a lowering of the mean blood pressure to 45 mm Hg disrupts energy balance in the brains of shunted rats

B *Reduction in blood pressure to 30 mm Hg* In this material the comparison was made between one shunted and one non shunted control group both bled to a mean arterial blood pressure of 30 mm Hg for 30 min The brain regions analysed were

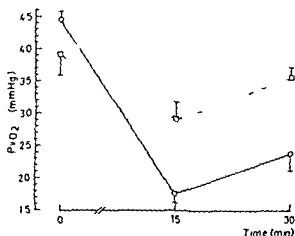



Fig 2 Oxygen tensions in cerebral venous blood of non shunted or shunted rats at a mean arterial blood pressure of 30 mm Hg Circles denote mean values for non shunted rats and squares for shunted rats Vertical bars denote ±S E (n=9)

the frontal lobe the brainstem and the parietal cortex with underlying white matter The latter structure was chosen because it includes the border zone between the distribution territories of the anterior and middle cerebral arteries Such border zone areas are known to show ischemic cell changes in hypotensive conditions (Brierley *et al* 1969)

In 9 out of 27 animals bled to a mean blood pressure of 30 mm Hg there were cardiac irregularities and heart standstill ɔ to 15 min after the bleeding was started These animals all died In the nonshunted group 7 out of 16 rats died while only two of the shunted animals died The difference in mortality occurred despite the fact that the maximal volume of blood that had to be withdrawn in order to maintain the blood pressure at 30 mm Hg was significantly larger in the shunted rats (8 7± 0 3 ml) than in the non shunted controls (6 4±0 3 ml p<0 01) Thus the shunted rats appeared to be more resistant to the bleeding than were the controls

Fig 2 shows that the venous $P_{O_2}$ was lower in the control group than in the shunted group both at 15 and 30 min (p<0 01) This difference occurred in spite of identical arterial $O_2$ tensions and could not be explained by difference in hemoglobin concentration or in plasma acid base parameters Thus the values indicate

TABLE 1 Ammonia contents in frontal lobe parietal cortex and brainstem in non shunted control animals non shunted hypotensive animals and shunted hypotensive animals Means± S E in mmol/kg wet wt n = number of rats

| Exp group | Frontal lobe | Parietal cortex | Brainstem |
|---|---|---|---|
| Control (n = 5–8) | 0 27 ±0 02 | 0 22 ±0 02 | 0.22 ±0 04 |
| Hypotension (n = 5–9) | 0 47 ±0 06 | 0 56 ±0 10 | 0.35 ±0 03 |
| Hypotension + P-c shunt (n = 5–9) | 0 84[1] ±0 09 | 0 8ɔ ±0 16 | 0 4 ±0 07 |

[1] significantly increased in relation to hypotensive control group (p < 0 05)

TABLE VI Contents of lactate and pyruvate as well as lactate/pyruvate ratios in different brain structures of control rats or shunted rats at a mean arterial blood pressure of 30 mm Hg n = 5—9 Means ± S E Contents in mmol/kg wet wt

| Exp group | LAC | PYR | LAC/PYR |
|---|---|---|---|
| *Frontal lobe* | | | |
| Controls | 10 07<br>±1 39 | 0 223<br>±0 008 | 44 7±5 4 |
| P-c shunt | 8 46<br>±1 75 | 0 197<br>±0 021 | 40 0±4 1 |
| *Parietal cortex* | | | |
| Controls | 10 10<br>±1 08 | 0 193<br>±0 009 | 53 3±6 2 |
| P-c shunt | 9 19<br>±1 49 | 0 190<br>±0 013 | 46 3±5 1 |
| *Brainstem* | | | |
| Controls | 7 20<br>±1 94 | 0 165<br>±0 023 | 39 8±5 6 |
| P c shunt | 5 57<br>±1 05 | 0 148<br>±0 020 | 36 3±2 4 |

that the blood flow was better upheld in the shunted animals or that shunted animals have a lower metabolic rate for oxygen than have non shunted controls (see Discussion)

Table V compares the ammonia contents in frontal lobe parietal cortex and brainstem in non shunted control animals in non shunted hypotensive animals and in shunted hypotensive animals The control values for frontal lobe and brainstem were taken from Holmin and Siesjo (1972 a) and for parietal cortex from MacMillan and Siesjö (1972) Hypotension in non shunted animals gave rise to increases in ammonia content in all three regions studied There was a further increase in ammonia content in the shunted animals but this was significant only in the frontal lobe A comparison with previous data for ammonia contents in frontal lobe and brainstem in shunted rats with a normal blood pressure (Holmin and Siesjo 1973 a) shows that the reduction of blood pressure to 30 mm Hg in the shunted rats also led to further increases in ammonia content (see Discussion)

Table VI shows the tissue lactate and pyruvate contents and the lactate/pyruvate ratios There were no differences in any of these variables between the non shunted and shunted animals Since the tissue lactate contents were similar in shunted animals and in non shunted controls and since the groups had similar tissue $CO_2$ tensions the changes in intracellular pH should have been about the same From this and from the observed lactate/pyruvate ratios we may conclude that the cytoplasmatic NADH/NAD ratios of the two groups must have been similar

Fig 3 illustrates the contents of carbohydrate substrates and aminoacids in the hypotensive non shunted and shunted groups For comparison all values have been given in percentage of the corresponding values measured in non shunted normotensive animals In the frontal lobe and parietal cortex the pattern of changes seen was very similar In the non shunted animals there was an increase in tissue glucose content while the shunted animals showed decreases in glucose content (*cf* Holmin

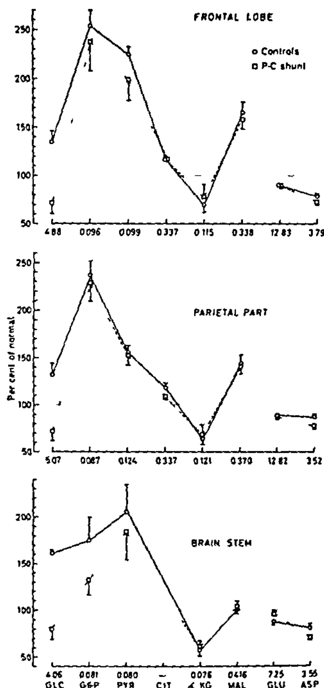



Fig 3 Contents of glucose (GLC) glucose 6-phosphate (G6P) pyruvate (PYR) citrate (CIT) α ketoglutarate (α KG) malate (MAL) glutamate (GLU) and aspartate (ASP) expressed as percentage of the values for the same metabolites in different brain structures of normotensive nonshunted rats The normal values for frontal lobe and brainstem are taken from Holmin and Siesjo (1973 a) with the exception of the citrate value This value and the values for parietal cortex are taken from Bachelard Lewis Ponten and Siesjo in preparation Vertical bars denote ± S E not shown if equal to or less than the diameter of the symbol The control values are mean values All values in mmol/kg wet wt in groups of 8—9 rats

and Siesjo 1972 a and b) However apart from this difference identical changes were seen in the shunted and non shunted groups These changes consisted of increases in glucose 6 phosphate pyruvate and malate and decreases in α ketoglutarate glutamate and aspartate

TABLE VII Contents of phosphocreatine ATP ADP AMP calculated adenylate energy charge (ECP) and adenylate sum (S) in different brain structures of control rats or shunted rats both groups bled to 30 mm Hg for 30 min Means±S E in mmol/kg wet wt n = number of rats

| Part of brain | Exp group | PCr | ATP | ADP | AMP | ECP | S |
|---|---|---|---|---|---|---|---|
| Frontal lobe | Control | 4.04 | 3.11 | 0.307 | 0.075 | 0.948 | 3.44 |
| | (n = 9) | ±0.11 | ±0.07 | ±0.006 | ±0.001 | ±0.001 | ±0.03 |
| | P c shunt | 3.96 | 3.09 | 0.311 | 0.079 | 0.946 | 3.44 |
| | (n = 9) | ±0.18 | ±0.05 | ±0.006 | ±0.002 | ±0.002 | ±0.05 |
| Parietal cortex | Control | 4.06 | 2.84 | 0.381 | 0.050 | 0.925 | 3.27 |
| | (n = 9) | ±0.18 | ±0.12 | ±0.070 | ±0.016 | ±0.010 | ±0.09 |
| | P-c shunt | 3.77 | 2.85 | 0.345 | 0.056 | 0.929 | 3.26 |
| | (n = 9) | ±0.22 | ±0.08 | ±0.016 | ±0.016 | ±0.008 | ±0.05 |
| Brainstem | Control | 4.00 | 2.64 | 0.236 | 0.077 | 0.950 | 2.91 |
| | (n = 6) | ±0.31 | ±0.06 | ±0.009 | ±0.002 | ±0.003 | ±0.06 |
| | P-c shunt | 3.83 | 2.58 | 0.252 | 0.031 | 0.945 | 2.86 |
| | (n = 5) | ±0.19 | ±0.05 | ±0.003 | ±0.002 | ±0.002 | ±0.05 |

In Fig 3 the shunted and control animals bled to a blood pressure of 30 mm Hg were compared to a normotensive control group and the values therefore do not illustrate the effect of the hypotension upon metabolite values in shunted animals We have previously demonstrated that the porta-caval anastomosis by itself leads to changes in the levels of some metabolites Thus the contents of glucose and aspartate decreased and there was a tendency for lactate to increase somewhat (Holmin and Siesjo 1973 a) When it is taken into account that these changes exist in the shunted animals before they are bled it is found that the hypotension led to moderate *increases* in glucose content and that the percentage increase in lactate was less in shunted animals than in the controls In the shunted rats and at least in the brain stem there were also less marked decreases in *α* ketoglutarate and aspartate following reduction in blood pressure than in the controls

Table VII shows the tissue contents of phosphocreatine ATP ADP and AMP the calculated adenylate energy charge and the sums of the adenine nucleotides in frontal lobe parietal cortex and brainstem The data demonstrate two things Firstly there were no differences in any of these parameters between the shunted and the control groups *i e* the shunting procedure did not adversely affect the energy state of the brain upon a reduction in blood pressure to 30 mm Hg Secondly both the control animals and the shunted ones showed regional changes in response to the bleeding In the frontal lobe the values for ATP ADP and AMP were similar to those previously measured in normotensive animals (see Table IV and Holmin and Siesjo 1973 a) Thus although the hypotension was associated with decreases in phosphocreatine and increases in lactate it was unaccompanied by detectable changes in adenylate energy charge In the parietal cortex and the brainstem however the values for ATP ECP and adenine nucleotide sum were lower and the values for ADP and AMP higher than those measured previously in control animals (MacMillan and Siesjo 1972 Folbergrova *et al* 1972 a) Although these changes suggest that the hypotension gave rise to a derangement of the energy state of the

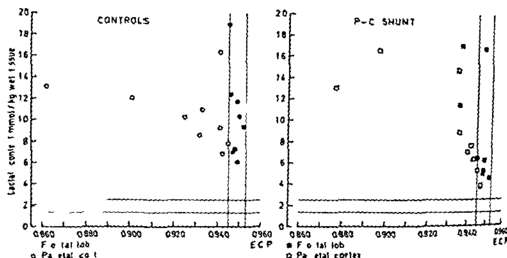



Fig 4 Relation between lactate content and energy charge potential (ECP) in the brains of shunted rats and of control rats exposed to hypotension (30 mm Hg) for 30 min

$$ECP = \frac{[ATP] + 0.5\,[ADP]}{[ATP] + [ADP] + [AMP]}$$

The drawn lines denote outer ranges for normal values of lactate content and ECP obtained in three recent materials from the laboratory

parietal cortex in shunted as well as in control animals marked changes in ATP and AMP were seen in only two animals in each group In Fig 4 the individual values for ECP were related to the corresponding lactate contents There was a gross relationship between decrease in ECP and increase in lactate However even in animals showing normal values for ECP the lactate contents were invariably increased

## Discussion

The objective of the present study was to evaluate the influence of arterial hypotension upon the energy metabolism of the brain in rats provided with a porta-caval anastomosis As stated in the introduction it has been assumed by many that liver failure and the accompanying hyperammonemia may induce encephalopathy via a toxic influence on energy production in the brain If this were so one would expect signs of a disruption of the balance between production and utilization of energy if the hyperammonemia is combined with any other measure that threatens the energy metabolism such as hypotensive ischemia The experiments were therefore devised to provide levels of arterial hypotension that are just about tolerated by the normal brain or that give relatively moderate signs of ischemic changes in cerebral metabolism When the mean arterial blood pressure is decreased to 30 mm Hg there are moderate to marked changes in contents of adenine nucleotides as well as of lactate and pyruvate (Reulen *et al* 1968 Kaasik *et al* 1969 Siesjo and Zwetnow 1970) but at 45 mm Hg most animals show a normal or nearly normal metabolic state unless the hypotension is combined with hypercapnia (Eklof *et al* 1972 Eklöf *et al* 1973)

These levels of hypotension were therefore studied in the present experiments Since cerebral ischemia is apt to develop in an inhomogeneous fashion (see Siesjo *et al* 1972) we abstained from attempts to calculate intracellular concentrations of lactate and pyruvate from the contents in tissue CSF and blood and intracellular pH changes were not derived

The present results failed to demonstrate that rats with a porta-caval anastomosis develop more pronounced changes in cerebral metabolism during hypotension than do non shunted animals At a mean blood pressure of 45 mm Hg the shunted animals had essentially unchanged contents of carbohydrate substrates amino acids and adenine nucleotides The data suggest that minor changes could have been present in glucose 6 phosphate and in lactate but since similar or larger changes are seen in non shunted animals at the same pressure (Eklof *et al* 1972) there were no alterations that could be ascribed to the porta caval shunt *per se*

Shunted animals bled to a mean blood pressure of 30 mm Hg showed less mortality than control animals and a larger volume of blood had to be withdrawn in the shunted animals in order to obtain a pressure of 30 mm Hg These animals were thus at least as resistant to bleeding as non shunted controls There was a striking similarity in cerebral metabolites in all three brain regions studied between the shunted and non shunted animals at identical blood pressure In fact since the porta-caval shunt by itself leads to moderate metabolic changes such as decreases in phosphocreatine and aspartate a tendency towards a fall in *a* ketoglutarate and suggested increases in lactate concentration and cytoplasmatic NADH/NAD ratio (Holmin and Siesjo 1972 a) the shunted animals showed less changes in hypotensive ischemia than did the controls

The symptomatology of hyperammonemic states indicates that brainstem functions may be specifically affected and experimental hyperammonemia has been found to be associated with slight decreases in brainstem contents of phosphocreatine and ATP (Schenker *et al* 1967 see also Hindfelt 1972) The present results demonstrate some specific features in brainstem metabolite concentrations as a result of the hypotension Thus there was a more pronounced fall in *a* ketoglutarate than in the other structures and no change in malate However since there was no difference in metabolite pattern between shunted animals and controls and since the adenine nucleotide concentrations did not differ we conclude that the porta caval shunt and the accompanying hyperammonemia did not adversely affect cerebral energy metabolism in any of the regions studied during the hypotensive stress

As remarked in the introduction hepatic encephalopathy is associated with a decreased cerebral metabolic rate for oxygen In the present experiments the hypotensive shunted rats showed a higher cerebral venous $P_{O_2}$ than the controls This finding is either compatible with a decreased cerebral metabolic rate or with a relative increase in cerebral blood flow in the shunted animal Either of these factors may evidently aid in protecting the tissue against the metabolic effects of arterial hypotension

The present results provide new information on changes in cerebral metabolism

during hypotensive ischemia in control animals. Firstly, although there was an increase in lactate and decrease in phosphocreatine in all three regions at a blood pressure of 30 mm Hg, only the parietal cortex showed clear changes in ATP, ADP and AMP. This may have been due to the fact that this region includes the border zone between the distribution territories of the anterior and the middle cerebral arteries that is prone to develop a more serious underperfusion than other areas (see Brierley *et al.* 1969). Secondly, although a significant fraction of the animals died when the mean blood pressure was reduced to 30 mm Hg, the majority of those which survived showed no or only very small change in adenine nucleotides in spite of relatively marked increases in lactate content. Since larger changes in ATP, ADP and AMP have previously been observed with hypotensive periods lasting 3 to 10 min (Kaasik *et al.* 1969), it is conceivable that regulatory mechanisms allow near normalization of the energy state when the hypotension is prolonged. Thirdly, the tissue changes observed with a reduction in blood pressure to 30 mm Hg are similar to those observed in normotensive hypoxemia, especially those observed after a reduction in arterial $P_O$ to below 20 mm Hg (MacMillan and Siesjö 1972). Thus both conditions lead to pronounced increases in lactate at normal or near-normal contents of ATP, ADP and AMP, and to increases in glucose and glucose-6-phosphate. This pattern is different from that obtained in total ischemia which is characterized by marked changes in adenine nucleotides and by decreases in glucose and G6P, i.e. by signs of substrate depletion (Lowry *et al.* 1964; Duffy *et al.* 1972; Eklöf and Siesjö 1972). Thus, as long as a decrease in blood pressure does not lead to total ischemia the tissue changes seem dominated by the relative oxygen lack. It must be stressed, though, that this conclusion applies to the metabolic pattern of relatively gross tissue regions. In pure hypoxia the cerebral venous $P_O$ can be reduced to below 10 mm Hg without giving detectable changes in adenine nucleotides (MacMillan and Siesjö 1972; Lewis *et al.* 1973). In the present experiments changes in adenine nucleotides were seen in parietal cortex at venous $P_O$ values exceeding *20 mm Hg. This suggests that regions of total ischemia may exist within the tissue* and that the changes observed in ATP, ADP and AMP contents may be secondary to regional nonperfusion.

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# Respiratory and Cardiovascular Responses to Electrical Stimulation of the Avian Brain with Emphasis on Inhibitory Mechanisms

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## Abstract

Kotilainen P V and P T S Putkonen *Respiratory and cardiovascular responses to electrical stimulation of the avian brain with emphasis on inhibitory mechanisms* Acta physiol scand 1974 *90* 358—369

Respiratory and circulatory responses to electrical brain stimulation were studied in 30 anesthetized chicken from about 850 histologically verified loci in an attempt 1) to delineate the limits and characterize the response patterns of a thalamic cardioinhibitory area (Putkonen 1967) and 2) to extend exploration of respiratory and circulatory functions of the avian brain to telecephalic and mesencephalic levels Apnea or partial respiratory inhibition were obtained from 1) an anterolateral thalamic field in front and around the nu rotundus 2) the entire course of the quinto-frontal tract which was the only forebrain site yielding apnea and from 3) a medial and a lateral mesencephalic area The apnea from the antero-lateral thalamus was associated with progressive bradycardia and fall in blood pressure which both could be almost abolished during tracheal infusion of oxygen Apneic bradycardia from the mesencephalic quinto-frontal tract was associated with hypertension thus mimicking the diving reflex Varying patterns of respiratory and circulatory activation often with vocalizations were obtained from 1) septal and preoptic areas 2) medial archistriatal regions 3) the occipito-mesencephalic tract and from 4) a large field in the mesencephalic lateral reticular formation The results are discussed in relation to pertinent literature from birds and mammals with an emphasis on inhibitory mechanisms

*Electrical stimulation of the brain (ESB) has been used extensively* to study central nervous control mechanisms of respiratory and cardiovascular functions in mammals (see *e g* Hess 1947 Kaada 1951, 1960 Oberholzer and Tofani 1960 Uvnäs 1960 Wang and Ngai 1964)

In birds Vediaev (1963 1964) has described respiratory responses to ESB from 5?

*Abbreviations* AC = commissura anterior AR = archistriatum AS = area septalis CHI = chiasma LFB = lateral forebrain bundle MLD = nu mesencephalicus *lateralis* pars dorsalis NE = neostriatum OM = tractus occipito-mesencephalicus PAM = paleostriatum augmentatum PALP = paleostriatum primitivum PC = commissura posterior *QF* = tr quinto-frontalis ROT = nu rotundus SM = tr septomesencephalicus SP = tr striopeduncularis (Papez) = tr occipito-mesencephalicus (Huber & Crosby) TTC = tectum opticum

loci mainly in striatal and thalamic levels of awake doves Richards (1971) stimulated the brain stem of pigeons and chicken concentrating on polypneic responses Peek and Phillips (1971) explored systematically the brain of anesthetized fowl for vocal responses They point out and discuss the obvious and extensive convergence of vocal and respiratory mechanisms (for vocalization see also Brown (1971) and Delius (1971)) Cardiovascular and respiratory effects from stimulating medullary vagal nuclei in the pigeon are described by Cohen and Schnall (1970) and some respiratory and circulatory data are also given by Cohen and Pitts (1967) from ESB in the hyperstriatum of pigeons Putkonen (1967) reported heart rate changes during the stimulation of the basal forebrain and the diencephalon in the chicken

The present experiments were initiated to analyze the circulatory and respiratory effects of ESB in a lateral thalamic field previously noted by one of us (Putkonen 1967) to yield in freely moving chicken a trophotrope (Hess 1947) stimulation syndrome consisting of crouching and motor inhibition often overlasting the stimulation and stimulus bound feather erection miosis and bradycardia Accordingly during the early part of the study when blood pressure was recorded most electrode tracts were concentrated to the lateral diencephalon A preliminary note based on this material has been published (Kotilainen and Putkonen 1972) At a later phase designed mainly to explore the extensions of the respiratory inhibitory area oral and caudalwards from the thalamus only respiration and pulse rate (ECG) were monitored

## Material and Method

Thirty adult white Leghorns were used for the experiments They were anesthetized with Nembutal® 40—60 mg/kg im In ten birds the sciatic artery was cannulated for blood pressure and pulse rate recording with an electromanometer (Sandborn Co 121 A) In 20 birds the heart rate was monitored by electrocardiography via thoraco-abdominal needle electrodes The respiratory movements were recorded with thoracometric transducer (a rubber tubing filled with saturated $CuSO_4$ around the thorax changing its resistance when stretched by inspiration) All records were obtained with an electroencephalograph (San Ei Type EG 900) using paper speeds of 2 mm/s and 15 mm/s for heart rate count The time constant for the respiratory record was 1 5 s In 4 birds the trachea was cannulated to test the effects of $O_2$ tracheal occlusion on the response others breathed naturally Steel electrodes (0 3 mm Ø) with 0 5 mm uninsulated tips were used for stereotaxic exploration guided by the co-ordinates of the van Tienhoven and Juhasz (1962) atlas From 2 to 6 tracts per bird were stimulated at 1—0 5 mm intervals usually beginning from horizontal level +10 The monopolar stimuli delivered from a constant voltage stimulator (Nihon Kohden Type MSE 3R with isolating unit) were trains of rectangular cathodal 2 ms pulses at a frequency of 50 Hz Intensities ranged from 200 μA to 400 μA (rarely up to 600 μA) The current was monitored by observing the potential across a 1 kΩ resistance in series with the stimulating circuit The indifferent electrode was a steel screw in the frontal spongiosa of the skull

Each electrode tract was marked at 1 to 3 horizontal levels electrolytically by passing DC current through the electrode serving as the anode The birds were killed with an overdose of Nembutal the brains perfused through the carotid arteries with 10 buffered formalin The electrode tracts were reconstructed from histological sections cut in the stereotaxic plane and stained alternating for nuclei and myelinated fibers The stereotaxic co-ordinates from the experimental protocol were corrected according to the electrolytic lesions and Prussian blue markings on the sections and projected on appropriate stereotaxic planes When a few tracts were excluded due to ambiguocity in histological verification or faults in electrode insulation the total number of stimulated loci was 850 At 380 of these arterial pressure was measured during stimulation

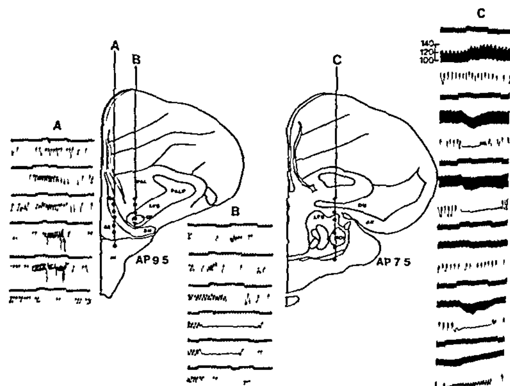



Fig 1 Respiratory and circulatory responses to ESB at successive points along three electrod tracts depicted on anterior levels 9.5 and 7 5 of the stereotaxic atlas The dots correspond to the compiled polygraphic records Stimulation is indicated on the time mark (1 s d Inspiration is upwards in all respirograms Blood pressure calibrations in mmHg In A a 400 μA ESB evokes hyperpneas with sharp rhythmic expirations (vocalizations) in the septal area. The slight accelerations of respiratory rhythm in the above two loci are classified as negative responses In B a 400 μA stimulus evokes two inspiratory apneas in an area sharply limited to the quinto-frontal tract A point in the paleostriatum augments (second from the top) gives a moderate increase in respiratory amplitude without cha the frequency In C a 200 μA ESB evokes apneas with a fall in blood pressure (and bradycardia) in two areas above and under but not in the nu.rotundus. The lowest re rated as respiratory inhibition.

## Results

The respiratory changes evoked by ESB were sometimes limited to the frequency or amplitude of respiration but more often both changed simultaneously Furthermore many responses with strong vocal components and various biphasic responses (e g Fig 3 A and B resulted in a variety of response patterns illustrated in Fig 1–4 To summarize the anatomy of the main respiratory findings the various responses have been classified into the following categories

1) *Hyperpneic responses* (203 loci) These include all the points giving marked polypnea (= an at least twofold increase in frequency) and/or a clear increase in amplitude (Fig 1 B second response from the top is at the acceptable l

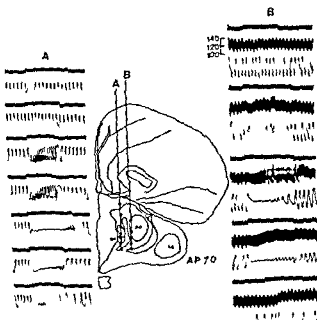



Fig 2 A Two apneas in the quinto-frontal tract and an inhibitory response below it to 300 μA ESB The two responses above QF are rated as hyperpneic in spite of a small initial diminution in amplitude At these points the frequency increased from *ca* 30/*min to* 160/*min* with small expiratory movements interspaced between higher excursions Rhythmic clucking was produced B Short respiratory arrest with some residual oscillations during 400 μA stimulation of an area ventromedial to the nu rotundus Note strong bradycardia (level 3 from bottom) ending abruptly with the ESB A moderate hypertensive effect accompanies these responses

according to the latter criterion) About a half (115/203) of the hyperpneic loci showed convergence of respiratory and vocal systems

2) *Respiratory inhibition* a) Apnea (131 loci) b) Partial inhibition (45 loci) a) For the apneas the criterion was an at least 5 s blocking of respiratory movements *Some* residual oscillations (under 10 % of prestimulus amplitude (*e g* Fig 2 B)

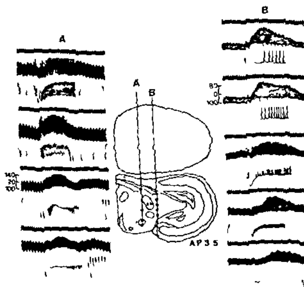
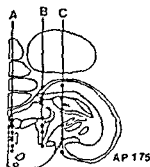



Fig 3 A Polypneic and hypertensive responses to 200 μA ESB from the mesencephalon with two short apneas first with initial polypnea) as the QF is traversed B Initial expiratory apnea with marked hypertension from a pretectal area under th posterior commissure (300 μA) Sharp inspiratory mo ments follow the in tial pa e and a prolonged insp r on follo s as an aft r effec f the ESB As the electrod is plunged d eper pol pn c pa tern with rhythm c b tory zations appears An nh response term nat s d



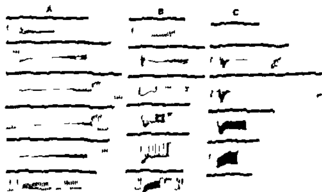




Fig 4 A Series of apneic responses in medial pontine area B Short apneas from dorsal pretectal loci changing to hyperpneas with vocalizations at deeper levels C Apneas (with initial polypnea) from the region of the MLD and beneath it and an area giving intense polypneas (up to 220/min) in lateral reticular formation All tracts in this figure are from the same bird and stimulus intensity 300 µA throughout

were allowed and cases with short initial polypnea (e g Fig 3 A) were included b) Partial inhibition was defined as an at least 5 s lasting 50 %—90 % reduction in the amplitude of the respirogram in the absence marked polypnea Thus e g the lowest response in Fig 3 B enters this class but the one immediately above with marked vocalization bound polypnea is classified as hyperpnea in spite of a ca 50 % reduction in amplitude

*c* 3 *Negative responses* (470 loci) These were either totally negative or gave in consistent responses or polypneas were too slight for criterion 1 (see Fig 1) These are not depicted in Fig 5

*Hyperpneic responses* form a very heterogeneous group which in Fig 5 are depicted with a single symbol mainly to provide a background for the more systematically studied inhibitory responses

The most anterior loci in the septal and preoptic areas were associated with either vocalization or gave a polypneic panting response with a moderate increase in frequency and some reduction in amplitude An increase in amplitude without a change in frequency was an infrequent observed response mainly in the paleostriatum augmentatum Increase in both amplitude and frequency was elicited from the medial archistriatum and the total course of the occipito mesencephalic tract at diencephalic and lower levels often in association with rhytmic vocalizations which were most intense in the posterior diencephalon around nu ovoidalis and in the mesencephalon in areas medial and ventral to the nu mesencephalicus lateralis dorsalis

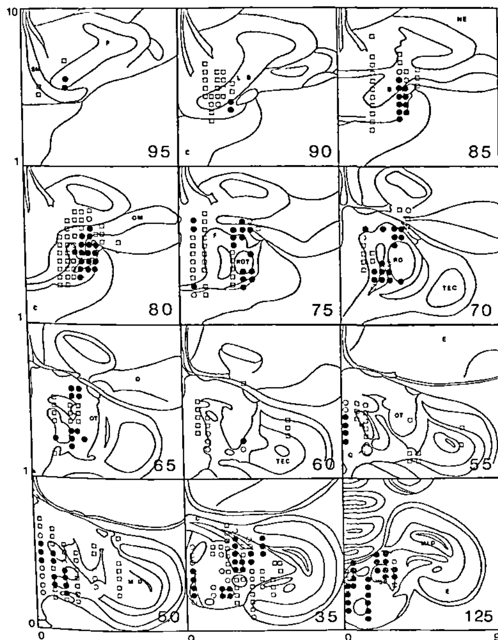



Fig 5 Respiratory eff cts of ESP ep e ented on frontal sections redrawn in s mplif ed form from the atlas of an 7 enho en and Juhasz (196?) □ = hyperpnea ● = apnea ○ = respiratory inhib tion (for crit ia se text) The number 9 5 to 1 ?5 refer to the stereotax plane (mm anterior to ear bar)

*Respiratory inhibition* Apneas and partial inhibition differed from each other mainly in degree Subthreshold ESB at an apneic locus could result in partial inhibition while from points of the latter category typically surrounding clusters of apneic loci a total block could not be elicited even with augmented intensities

With the exception of a dorsal mesencephalic area (see Fig 3 B) where the respiration was initially blocked by an expiratory spasm (deformed by the time constant) followed by inspiratory gasps and an inspiratory spasm at the termination of the LSB the apneic response showed a rather homogenous pattern Respiration was usually stopped at the end of a normal inspiration or to the neutral position During sustained stimulation the respiratory amplitude was progressively resumed in spite of continued ESB The length of the attained apnea (see Fig 1 C) was a function of stimulation intensity the effectiveness of the particular site and other factors such as recent stimulation history and changing of the respiratory gases The longest apnea from the diencephalon was 44 s while breathing air and 160 s under oxygen Apneas up to 65 s one even ending fatally were obtained from the hind brain

The anatomical distribution (Fig 5) of the apneic (and partially inhibitory) loci shows a continuous field in the lateral thalamus anterior to and surrounding the nu rotundus which itself gave only negative responses The only telencephalic extension of this area found in the present study was delineated to the quinto-frontal tract (see Fig 1) which gave apneas also along its total course through the meso-diencephalic brainstem (Fig 2 A and 3 A) In the anterior half of the area studied a few apneic points were encountered outside the continuous lateral field in the area septalis and at the level of the supraoptic decussation (Fig 5 AP 7 5)

More caudally beginning from AP 5 5 there was a continuous midline area giving rise to long apneas A special group of expiratory apneas with bi or even triphasic response patterns (Fig 3 B and 4 B and C) was encountered more laterally in the mesencephalic pretectal area A few points bordering or even in the nu mesencephalicus lateralis dorsalis (Fig 4 C) yielded long apneas with initial polypnea This nucleus however, also contained some clearly negative points

*Cardiovascular effects* In the 19 birds (mean weight 1 76 kg) where the arterial pressure was monitored from the sciatic artery the average resting values were 115/90 mmHg at the beginning and 135/100 mmHg at the end of the experiments The respective heart rates were 315/min and 350/min The small changes may reflect a lighter anesthetic level towards the end of the experiment in spite of additional doses of Nembutal when required The pressure maxima reached during ESB were about 190/120 mmHg and the minima about 90/60 mmHg The maxima and minima in the heart rate ranged from 420/min to 130/min

Fig 6 indicates the anatomical distribution of the main cardiovascular findings The figure is limited posteriorly to the frontal plane AP 6 5 because arterial pressure recording was only occasionally extended to more caudal levels of stimulation In Fig 6 the criteria for a pressure change is an at least 20 mmHg change in either systolic or diastolic pressure and for a heart rate change the limit

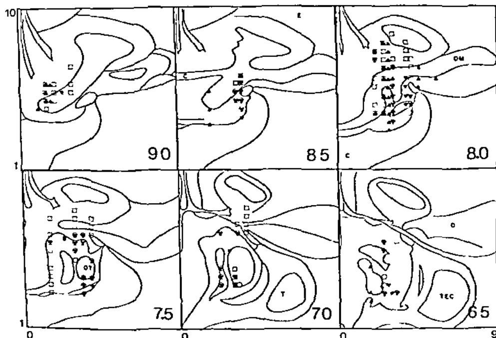



Fig 6 Cardiovascular effects of ESB □ = hypertensive response O = hypotensive response ▲ = tachycardia, ▼ = bradycardia. Responses from planes 9 5 and 6 5 have been added to the first and the last planes according to their lateral (0—9) and horizontal (1—10) co-ordinates

of acceptance was 20/min for both brady and tachycardia Struggling and vocalizations often obscured the heart rate record especially in cases where only the ECG was recorded resulting in obvious underpresentation of tachycardia in Fig 6

Pressor effects and tachycardia were the main responses from the area septalis paleostriatum augmentatum archistriatum mediale and tractus occipito-mesencephalicus Bradycardia and hypotension developing progressively during respiratory arrest (see Fig 7) were obtained from the apneic area in the lateral diencephalon At the dorsal or ventroposterior limits of this field bradycardia was occasionally coupled with moderate rise in arterial pressure (Fig 2 B) whereas apneic points in the mesencephalic part of the tractus quinto-frontali yielded marked pressor effects with bradycardia (Fig 3 A)

In four birds the trachea was cannulated to study the possible importance of hypoxia to slowing of the heart rate during apnea (Fig 7) Tracheal infusion of oxygen practically abolished bradycardia which was greatly enhanced by airway occlusion Obviously hypoxia is an important link in the bradycardia response Occlusion of trachea without stimulation however did not give similar results but rather an initial tachycardia linked with struggling which was inhibited during airway closure under ESB

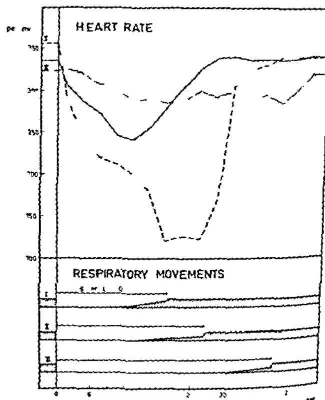



Fig. 7. The effects of respiratory manipulations on heart rate and respiratory movements *during 300 μA stimulation* of a point in the anterolateral thalamus. I (solid line) while breathing air. II *(broken line)* when the tracheal tube is occluded during ESB. III (pointed line) during tracheal infusion of O. *The bradycardia is practically abolished by O₂ in spite of a considerably longer lasting inhibitory effect on respiratory movements. Airway occlusion* on the contrary greatly enhances bradycardia.

*Associated responses.* In keeping with results from unanesthetized chicken (Putkonen 1967) the apneic responses from the anterolateral thalamus were typically accompanied by tonic erection of the feathers and pupillary miosis which was stronger in the contralateral eye. These effects did not accompany apneas from *more posterior levels. Intense hyperpneic and vocal responses were* often accompanied by eye opening and struggling.

## Discussion

The various hyperpneic responses to ESB have been contrasted in Fig. 5 with respiratory inhibition as a single group regardless of their great variability illustrated in Fig. 1—4. Some hyperpneic patterns such as polypneic panting are primarily concerned with thermoregulation, others appear mainly vocal while many responses especially from the hindbrain are more specifically respiratory.

Polypneic patterns with or without vocalizations were elicited by us from the *preoptic and septal area,* where Åkerman *et al.* (1960) and Richards (1971) evoked polypnea in pigeons. At variance with our results, Richards did not obtain polypnea from this anterior field in anesthetized chicken, which might be explained by a deeper anesthetic level, especially since Peek and Phillips (1971) in agreement

with us evoked vocalizations from the region in anesthetized chicken Polypneas not associated with vocalization were often of the thermal panting type with decreased amplitude In the medial archistriatum and adjacent sites in the occipito mesencephalic tract and the paleostriatum augmentatum increased respiratory amplitudes accompanied polypnea Tachycardia and hypertension were also frequently associated with these responses in accordance with earlier observations from this area yielding defensive behavioral reactions in awake chicken (Putkonen 1967) Polypneic and vocal responses with circulatory activation were obtained also from the whole diencephalic course of the occipito mesencephalic tract

A very powerful polypneic area was encountered by us in the mesencephalic portion of lateral reticular formation below the MLD in agreement with the results of Richards (1971) and Peek and Phillips (1971) who obtained intense repeated vocalization from this area in anesthetized fowl As discussed by the above authors this area may correspond to mammalian pneumotaxic center (Lumsden 1923) reported to respond by tachypnea to ESB (Ngai and Wang 1957) The expiratory apneas followed by inspiratory gasping (Fig 3 B) are reminiscent of responses described from lower medullary levels (nu reticularis parvocellularis) by Peek and Phillips (1971) *i e* protracted calls with expiratory apnea with suprathreshold ESB and repetitive calls with lower intensities

*Respiratory inhibition* with progressive bradycardia and hypotension was obtained by us from an anterolateral thalamic field in analogy with the results of Hess (1947) who described an trophotrope endophylactic zone yielding largely similar cardiovascular and respiratory effects in the lateral thalamus and subthalamus of the cat Feather erection and miosis as earlier described in awake chicken (Putkonen 1967) were also seen under anesthesia A few apneic points in Vedyaev s (1964) study in awake pigeons fall within this thalamic area according to Fig 1 in his paper Because of the complex anatomy of this region the effect is not easily pinpointed to any particular structure

The sharp delimination of respiratory arrest into the quinto-frontal tract through the entire anteroposterior extent of the area explored by us strongly implicates the participation of a trigeminal reflex mechanism in the respiratory inhibition The QF is an ascending direct path from the main trigeminal sensory nucleus to the nucleus basalis *in the basal anterior forebrain (Kappers et al 1960 p 1055* Karten 1969) If as proposed it is an exclusively afferent tract the respiratory arrest should be mediated *via* nu basalis which in turn projects to the dorsolateral nucleus of archistriatum and the overlying neostriatum (Karten 1969) These areas did not however give apneas in the present study although they readily yielded bill rattling and salivation pointing to trigeminal effects in awake (Putkonen 1967) and even anesthetized chicken It is conceivable nevertheless that the respiratory effects could be mediated through these areas and the lack of respiratory responses to local stimulation could be attributable to a heterogeneous cell population leading into failure to activate a sufficient amount of functionally related neurons perhaps more easily achieved indirectly via QF where a more homogeneous population of fibers

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# Denervation Changes in Frog Skeletal Muscle

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## Abstract

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The effects of denervation on acetylcholine sensitivity and on the electrical properties of frog tonic and phasic muscle fibres were examined by the use of iontophoretic micro-application of acetylcholine and conventional intracellular techniques for recording and stimulation. In tonic fibres extrajunctional cholinergic sensitivity appeared 4 days after sectioning of the nerve and by 2 weeks the entire membrane had attained a high level of acetylcholine sensitivity. The sensitivity of former end-plate regions also increased with time. In phasic fibres cholinergic sensitivity appeared about one week later but otherwise followed the same pattern as in tonic fibres. Innervated tonic fibres are incapable of generating action potentials but this property is induced upon denervation. After one week of denervation small spikes were observed in response to depolarizing current pulses and by 2 weeks large action potentials, sometimes followed by repetitive discharges, were recorded. Denervation failed to affect the action potential in phasic fibres. Tetrodotoxin completely blocked action potentials in tonic and in phasic fibres. Denervation reduced the effective resistance and the time constant of tonic fibres but had no effect on these properties in phasic fibres. Actinomycin D injected i.p. in a single dose (7.5 or 9.0 µg) *failed to prevent the appearance of extrajunctional cholinergic receptors but almost completely* blocked the induction of action potentials in denervated tonic fibres.


It is well established that in mammalian skeletal muscle denervation markedly increases the sensitivity of the muscle membrane to applied acetylcholine (ACh) and also alters the electrical properties of the muscle cell. A few days after denervation cholinergic receptors appear outside the former end-plate region and shortly thereafter the entire muscle membrane acquires a high sensitivity to applied ACh (Ginetsinsky and Shamarina 1942, Axelsson and Thesleff 1959). At the same time the resting membrane potential is reduced, the effective resistance of the cell is increased, the rate of rise of the action potential is reduced and the spike becomes resistant to the blocking effect of tetrodotoxin (Thesleff 1963, Albuquerque and Thesleff 1968, Redfern and Thesleff 1971 a and b).

Much less is known about the effects of denervation on frog skeletal muscle which in addition to phasic fibres (fast or twitch fibres) also contain tonic (or slow) fibres

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(Tasaki and Mizutani 1944 Kuffler and Gerard 1947 Zhukov and Leushina 1948 a) Tonic fibres differ from phasic ones by being multiply innervated (Kuffler and Vaughan Williams 1953) and being able to maintain tension during prolonged de polarization (Lüttgau 1963 Nasledov Zachar and Zacharova 1966 Lannergren 1967) In their electrical properties they differ by being incapable of generating action potentials (Kuffler and Vaughan Williams 1953 Burke and Ginsborg 1956) and by having a higher effective resistance and longer electric time and space constants than phasic fibres (Burke and Ginsborg 1956 Adrian and Peachey 1965 Stefani and Steinbach 1969)

In phasic muscle fibres Miledi (1960) has shown that following denervation the area of the membrane ensitive to ACh increases and involves after some 10 weeks the whole fibre surface In tonic fibres Miledi Stefani and Steinbach (1971) have observed that following denervation the fibres acquire the ability to generate action potentials Little is known about the appearance of other denervation changes and therefore in the present study we have investigated in more detail the effects of denervation on frog muscle with emphasize on tonic fibres their sensitivity to applied ACh and on alterations in their electrical properties

## Methods

The experiments were made on the isolated iliofibularis and pyriformis muscles of English male frogs (*Rana temporaria*) during the period of November—February The muscles were surgically denervated on the right s de of the animals under ether anesthesia by sectioning the nerve branch close to the muscle After the operation the frogs were kept at room temperature

For the electrophysiological study the pyriformis muscle or the surgically isolated tonus bundle (Sommerkamp 1928) of the iliofibularis muscle were mounted in a Perspex bath containing normal Ringer solution (mM) NaCl 115 5 KCl 2 0 CaCl 1 8 $NaH_2PO_4$ Na HPO₄ buffer ?0 pH 7 0 kept at room temperature (20—22 C)

Conventional intracellular techniques for recording and stimulation were used Tonic and phasic muscle fibres were distinguished by the d fferent electrical characteristics of their membranes (Burke and Ginsborg 1956 Adrian and Peachey 1965 Stefani and Steinbach 1969) The effective or input resistance and the time constant of single fibres were obtained by passing a rectangular hyperpolarizing current pulse of 2—3 s duration in tonic fibres and 100—200 ms duration in phasic fibres producing 2—5 mV potential change As time constant was taken the time for the anodal potential to reach 84 of its maximum steady phase

The ACh sensitivity of single muscle fibres was determined by iontophoretic micro-application of the drug and the intracellular record ng of the resulting membrane depolarization The procedure was to move the ACh containing micropipette along the surface of single fibres in steps of 50—300 $\mu m$ The recording electrode was always kept within 1 mm f om the ACh pipette The ACh sensit vity was exp essed as units one unit be ng 1 mV depolarization produced by $10^{-9}$ Coulombs passed th ough the pipette All the responses were corrected to a membrane potential value of −60 mV for tonic f bres and of −90 mV for phasic fibres (Katz and Thesleff 1957) As equil brium potent al for th ACh response in tonic fibres was taken −8 mV (Magazan k and Nasledo 1971) and in phasic fibres −15 mV (del Castillo and Katz 1954) In tonic fibres e eral peaks of ACh sensit ty could be observed representing the different end plate reg ons Each peak was included in the estim tes of mean maximal ACh sensitivity Minimal ACh sensit ity was the lowest sensitivity recorded n a fibre Average ACh sensitivity was obtained from th sen ti it e estimated for spots at 0 2 mm distance from each other along the fibre surfa e

Action potentials were generated and recorded us ng a double microelectrode technique The current passing electrod f lled with 2 M potassium citrate was inserted into the same fibre as the reco ding electrode at a sep at on of 50—100 $\mu m$ To ensure optimal conditions for act on potential generation the mu cle fibre was hyperpolarized to a local potential of −90—100 mV (Redfern and Thesleff 1971 a The time d fferential of the action potential was obtained by using a CR c rcu t Wl en tetrodotoxin was used it was added to the external

TABLE I Electrical properties of tonic muscle fibres at various times after denervation and the effects of actinomycin D The values are the mean ±S E Figures in brackets note the number of fibres examined

| Days of denervation | Resting membrane potential (mV) | Effective resistance (MΩ) | Time constant (ms) | Presence of action potential *) |
|---|---|---|---|---|
| 0 | 60 9±2 68 (9) | 6 2±1 73 (6) | 709±227 7 (6) | 0/6 |
| 3–6 | 55 7±2 91 (9) | 3 3±0 68 (9) | 480±97 1 (9) | 0/9 |
| 7–10 | 59 8±2 12 (11) | 3 3±0 50 (16) | 415± 63 0 (16) | 13/20 |
| 11–14 | 53 8±2 32 (16) | 3 7±0 62 (15) | 452± 64 6 (15) | 17/17 |
| 15–20 | — | 1 8±0 46 (8) | 236± 48 4 (9) | 9/9 |
| 21–35 | 59 6±3 33 (10) | 2 9±0 90 (7) | 277± 54 1 (8) | 13/13 |
| | After actinomycin D treatment | | | |
| 9–17 | 65 7±2 28 (20) | 4 4±0 50 (18) | 624± 57 1 (18) | 2/16 |

* Number of fibres responding / number of fibres examined

solution to give a concentration of $10^{-7}$—$10^{-6}$ M and at least 15 min was allo ed for equilibration to take place

Some of the frogs received a single i p injection of actinomycin D (7 5 or 9 0 μg) 2 days after denervation

Actinomycin D was obtained from Sigma Chemical Company Illinois and tetrodotoxin 3 × crystalline from Sankyo Company Ltd Tokyo All other drugs and chemicals were obtained from British Drug House Poole England

## Results

*Electrical properties* Denervation reduced the effective or input resistance of tonic muscle fibres as shown by the values in Table I From about 6 MΩ in innervated fibres the resistance fell apparently within the first 3 days to about one half Similarly the electrical time constant of the muscle membrane was affected From about 700 ms in innervated fibres the time constant following denervation was reduced to about 250 ms The resting membrane potential however was unaffected by denervation remaining throughout the period at the level of −55—60 mV

In agreement with previous studies (Kuffler and Vaughan Williams 1953 Burke and Ginsborg 1956 Orkand 1963 Stefani and Steinbach 1969) we found that innervated tonic muscle fibres were incapable of generating action potentials We also confirmed a was originally described by Miledi Stefani and Steinbach (1971) that denervation induced the generation of action potential in tonic fibres 6 days after denervation an electrotonic potential produced by a depolarizing current pulse of adequate strength howed some signs of regenerative response and by 7 to

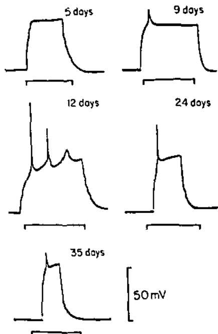



Fig 1

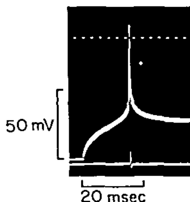


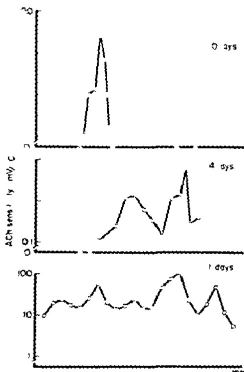

Fig 2

Fig 1 Typical records of spike potentials recorded 9 12 24 and 35 days after denervation in tonic fibres 5 days after denervation the fibre failed to respond by a regenerative response Time scale 2 s

Fig 2 Action potential (upper tracing) and its first derivative (lower tracing) in a 13 days denervated tonic fibre The dotted line indicates the zero potential level of the cell Voltage calibration equals a rate of rise of 500 V/s

10 days a small spike potential was observed in 10 out of 20 fibres (see Fig 1 and Table I) By 11 days a depolarizing current pulse of threshold strength invariably produced an action potential as shown by Table I and Fig 2 In 7 fibres a repetitive discharge was observed during the period of depolarization as shown in the tracing from a 12 days denervated muscle in Fig 1 After 15 days of denervation repetitive action potentials were recorded in only one fibre

The rate of rise of action potential varied between fibres from values too slow to be accurately measured to values as high as 50 V/s The amplitude of the action potential similarly varied only occasionally (in 4 fibres) did the potential exceed the zero level of the cell the maximal amount of overshoot observed was 10 mV (Fig 2) Since the action potential of mammalian muscle after denervation becomes resistant to the blocking effect of tetrodotoxin (Redfern and Thesleff 1971 b) we decided to examine if a similar resistance to the drug was induced in tonic fibres Tetrodotoxin in a concentration of $10^{-6}$ M completely abolished action potentials during the whole period of denervation With $10^{-7}$ M of tetrodotoxin some signs of a regenerative response remained in fibres denervated for 12—15 days



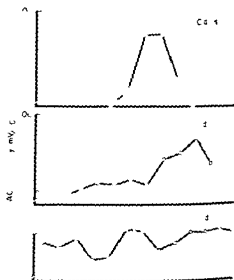



Fig 3

Fig 4

Fig 3 Distribution of ACh sensitivity in individual tonic fibres 0 4 and 11 days after denervation Abscissa indicates distance along the fibre surface in mm

Fig 4 Distribution of ACh sensitivity in individual phasic fibres 0 13 and 24 days after denervation

*ACh sensitivity* In tonic fibres which are multiply innervated spots of ACh sensitivity corresponding to end plate regions are observed along the entire length of the fibre at distances of about 1 mm from each other (Nasledov 1969) Between the end plates the muscle membrane lacks ACh sensitivity as is shown in the upper part of Fig 3 Following denervation ACh sensitivity starts to appear in the areas between end plates and already 4 days after sectioning of the motor nerve the areas sensitive to ACh merge and 1 week later the entire muscle membrane has attained a high level of ACh sensitivity even if it still retains its peaks of maximal ACh sensitivity (Fig 3)

Table II shows the mean maximal (peak) minimal and average ACh sensitivities recorded in tonic fibres at various time after denervation Even if the sensitivity varied considerably between fibres it is obvious that maximal sensitivity i e presumably the sensitivity of the areas of the former end plates increased gradually from a mean of 13 units in innervated muscles to a mean of 87 units ($p < 0.005$) 5 weeks following denervation At the same time the minimal ACh sensitivity increased from zero value in innervated muscles to a mean of 11 units 3—5 week

TABLE II ACh sensitivity of tonic muscle fibres at various times after denervation and the effects of actinomycin D The values are the mean ± S E Figures in brackets note the number of fibres examined

| Days of denervation | Maximal ACh sensitivity mV/nC | Minimal ACh sensitivity mV/nC | Average ACh sensitivity mV/nC |
|---|---|---|---|
| 0 | 12 7 ± 6 33 (7) | 0 | — |
| 3–6 | 18 0 ± 9 79 (12) | < 0 01 | — |
| 7–10 | 23 3 ± 5 77 (14) | 0 6 ± 0 10 (6) | 8 3 ± 3 60 (5) |
| 11–14 | 44 0 ± 11 21 (15) | 4 1 ± 1 55 (5) | 18 7 ± 7 08 (6) |
| 15–20 | 71 9 ± 31 25 (12) | 5 9 ± 3 43 (7) | 28 0 ± 17 81 (8) |
| 21–35 | 87 3 ± 21 96 (20) | 10 7 ± 2 20 (9) | 36 1 ± 10 47 (9) |
| | After actinomycin D treatment | | |
| 12–17 | 39 4 ± 19 28 (11) | | 10 0 ± 4 29 (8) |

after denervation Correspondingly was the average sensitivity of all the points examined in a fibre increasing with time of denervation

*Phasic fibres*

*Electrical properties* Unlike tonic muscle fibres denervation had no apparent effect on the electrical properties of phasic fibres As shown by the values in Table III the resting membrane potential remained at about −80 mV the input resistance between 0 7 and 1 1 MΩ and the time constant between 34—44 ms

In mammalian skeletal muscle denervation induces a fall by about 1/3 in the rate of rise of the action potential and at the same time the action potential becomes partly resistant to the blocking action of tetrodotoxin (Redfern and Thesleff 1971 a and b) It was therefore of interest to determine whether denervation of frog phasic muscle fibres would similarly alter the action potential The mean rate of rise of action potentials in innervated phasic fibres was 262 V/s and in fibres denervated for 7 and respectively 14 days 303 V/s and 241 V/s as shown in Table III Tetrodotoxin in a concentration of $10^{-7}$ M completely blocked regenerative responses in innervated and in denervated fibres It thus appears that the changes in the action potential that are induced by denervation in mammalian skeletal muscle fail to develop in phasic fibres of the frog

*ACh sensitivity* An innervated phasic muscle fibre is sensitive to ACh only in an area restricted to and closely surrounding the neuromuscular junction (Miledi 1960) The total length of this region varies from fibre to fibre and averages about 1 mm

TABLE III Electrical properties of phasic muscle fibres from m. pyriformis at various times after denervation and the effects of actinomycin D. The values are the mean ± S.E. and the figures in brackets note the number of fibres examined

| Days of denervation | Resting membrane potential (mV) | Effective resistance (MΩ) | Time constant (ms) | Rate of rise of action potential (V/s) |
|---|---|---|---|---|
| 0 | 82.0 ± 1.87<br>(24) | 1.1 ± 0.41<br>(5) | 39.5 ± 8.39<br>(4) | 262 ± 16.6<br>(19) |
| 3–6 | 83.1 ± 2.06<br>(18) | – | – | – |
| 7–10 | 83.5 ± 1.14<br>(13) | 0.7 ± 0.09<br>(6) | 34.0 ± 7.58<br>(6) | 303 ± 14.7<br>(26) |
| 11–14 | 80.4 ± 1.80<br>(15) | 0.9 ± 0.15<br>(9) | 40.2 ± 8.02<br>(9) | 241 ± 11.5<br>(19) |
| 15–20 | 80.4 ± 3.51<br>(9) | 0.7 ± 0.14<br>(7) | 41.0 ± 11.34<br>(7) | – |
| 21–35 | 81.5 ± 2.29<br>(10) | 0.8 ± 0.21<br>(4) | 44.3 ± 11.93<br>(4) | – |
| | After actinomycin D treatment | | | |
| 8–17 | 81.3 ± 2.97<br>(20) | 0.8 ± 0.16<br>(10) | 23.6 ± 4.13<br>(7) | – |

TABLE IV ACh sensitivity of phasic muscle fibres from m. pyriformis at various times after denervation and the effects of actinomycin D. The values are the mean ± S.E. and the figures within brackets note the number of fibres examined

| Days of denervation | Maximal ACh sensitivity mV/nC | Minimal ACh sensitivity mV/nC | Average ACh sensitivity mV/nC |
|---|---|---|---|
| 0 | 30.6 ± 13.39<br>(11) | 0<br>(11) | – |
| 3–6 | 37.3 ± 11.67<br>(14) | 0<br>(14) | – |
| 7–10 | 40.5 ± 13.58<br>(8) | 1.4 ± 1.06<br>(5) | 9.1 ± 4.65<br>(5) |
| 11–14 | 56.7 ± 26.07<br>(13) | 1.5 ± 0.78<br>(5) | 14.0 ± 4.60<br>(4) |
| 15–20 | 39.9 ± 11.93<br>(9) | 2.5 ± 1.07<br>(9) | 10.0 ± 3.80<br>(7) |
| 21–35 | 137.2 ± 46.76<br>(12) | 19.5 ± 7.00<br>(9) | 72.9 ± 13.65<br>(7) |
| | After actinomycin D treatment | | |
| 8–11 | 29.3 ± 10.74<br>(4) | 0<br>(4) | – |
| 12–17 | 37.0 ± 24.08<br>(9) | 0.1 ± 0.06<br>(7) | 4.8 ± 2.65<br>(5) |

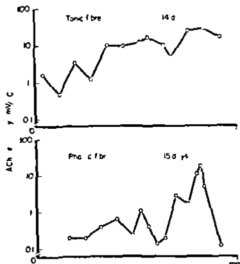



Fig 5 Distribution of ACh sensitivity in individual tonic and phasic fibres 14 and 15 days after denervation respectively The frogs received actinomycin D on the second day after denervation

as shown in Fig 4 Miledi has shown that following denervation the length of the muscle membrane sensitive to ACh increases in length In our experiments we observed that 13 days after sectioning of the nerve a large area of the extrajunctional membrane had a low level of ACh sensitivity Three to 4 weeks after denervation the ACh sensitivity of extrajunctional membrane areas had greatly increased and approached in some fibres that of the end plate zone (Fig 4)

Table IV shows the mean maximal minimal and average ACh sensitivities of phasic muscle fibres at various times after denervation From the values it appears that denervation not only induced extrajunctional ACh sensitivity but also increased the maximal ACh sensitivity that could be recorded in a fibre This was particularly prominent 3—5 weeks after denervation when peaks a maximal sensitivity averaging 137 units were present as compared to only 30 units in innervated muscles however this difference was only significant at the level of $p < 0.5$

*Effects of actinomycin D*

In mice the administration of actinomycin D in a dose sufficient to partially supress the *in vivo* synthesis of muscle RNA blocks the appearance of extrajunctional cholinergic receptors and of tetrodotoxin resistant action potentials in denervated muscles (Grampp Harris and Thesleff 1971) It was of interest to examine whether actinomycin D when given to frogs would similarly inhibit the development of denervation changes

Actinomycin D was injected i p in one dose of either 7 5 $\mu g$ or 9 0 $\mu g$ amounts which the majority of the frogs survived for 2 weeks Furthermore 7 5 $\mu g$ of actinomycin has been shown to block by 70—90 % the incorporation of the RNA precursor uridine into frog muscle (Bevan Miledi Grampp 1973)

Table I and III show that actinomycin treatment failed to affect the electrical properties of tonic and phasic muscle fibres with the possible exception that the fall in effective resistance and the reduction of the time constant that were observed in tonic fibres after denervation were less conspicuous in animals treated with actinomycin

The most striking effect of actinomycin was that it almost completely prevented the induction of action potentials in denervated tonic fibres While action potentials after 10 days of denervation invariably were elicited in tonic fibres from untreated frogs only two fibres with a small regenerative response were observed out of 16 muscle fibres examined in frogs denervated for 9—17 days and treated with actinomycin (Table I)

The development of extrajunctional ACh sensitivity in denervated tonic and phasic fibres was not prevented by actinomycin The magnitude of the sensitivity increase appeared however to be less after actinomycin treatment as shown by Fig 5 and by Table II and IV

## Discussion

The results of the present study show that denervation affects the physiological properties of frog skeletal muscle in a manner which in several aspects is similar but in others markedly different from that of mammalian muscle

In tonic fibres new ACh receptors developed during the first week of denervation as evidenced by the finding of a low level of ACh sensitivity in areas between the original end plates In contrast to what is observed in mammalian muscle (Axelsson and Thesleff 1959 Albuquerque and Thesleff 1968) the maximal sensitivities recorded were also increased and were about doubled in two weeks indicating that the process of receptor induction was not limited to extrajunctional regions but also *affected former end plates In phasic fibres extrajunctional cholinergic sensitivity* appeared later than in tonic fibres i e during the second week following denervation In contrast to Miledi (1960) we failed to observe a centrifugal spread of receptors from the end plate towards the tendons the new receptors instead appeared *along the entire cell surface which sensitivity to applied ACh increased with time* In this respect the appearance of cholinergic sensitivity was similar to that observed in rat skeletal muscle by Albuquerque and McIsaac (1970) It should be mentioned that phasic fibres of the pyriformis and iliofibularis muscles may have properties somewhat different from those of the sartorius muscle studied by Miledi (1960) since it is known that these muscles contain two kinds of twitch fibres ordinary phasic fibre and transitional fibres (Zhukov and Leushina 1918 b) As in tonic fibres denervation not only induced extrajunctional cholinergic sensitivity but also enhanced the sensitivity of the denervated end plate to close application of ACh even if this effect due to the wide variations in sensitivity among fibres was barely significant

In tonic fibres action potentials appeared 7—10 days after denervation Initially the spikes were small but the amplitude increased with time and 12—15 days after denervation large spikes and in some instances repetitive discharges were observed After 4—5 weeks of denervation the spikes again were smaller in amplitude indicating a reduction in the efficacy of the action potential generating mechanism In phasic fibres denervation failed to affect the action potential the rate of rise remaining at the level observed in innervated fibres This was in marked contrast to mammalian fibres in which denervation reduces the rate of rise of the action potential by one third (Redfern and Thesleff 1971 a) Another difference was the tetrodotoxin completely blocked action potentials in denervated frog muscle while in denervated mammalian muscle the action potentials are resistant to the blocking effect of this drug (Redfern and Thesleff 1971 b)

In mammalian skeletal muscle the resting membrane potential falls after denervation (Thesleff 1963) while in the frog we failed to observe such a change The effective resistance is increased after denervation in mammalian muscle but was decreased in frog tonic fibres and unchanged in phasic fibres Thus it appears that the effects of denervation on the electrical properties of frog muscle are different from those produced in mammals

Actinomycin D which is considered to block DNA dependent RNA synthesis (transcription) without affecting the translation stage of protein formation (Reich Cerami and Ward 1967) had little effect on the development of cholinergic sensitivity in denervated frog muscles but almost completely blocked the appearance of action potentials in denervated tonic fibres A similar treatment in mice completely blocks the appearance of new cholinergic receptors and of TTX resistant action potentials (Grampp Harris and Thesleff 1972) Thus it seems that in the frog the events which after denervation lead to the formation of new receptors and of action potentials in tonic fibres have a different sensitivity to actinomycin D and that therefore the mechanisms of protein synthesis that are involved in the appearance of the e denervation changes differ

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Acta physiol scand 1974 90 381—386

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# Pharmacological Exposure of Components in the Autonomic Control of the Diving Reflex

By

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## Abstract

ANDERSEN H T and A S BLIX *Pharmacological exposure of components in the autonomic control of the diving reflex* Acta physiol scand 1974 90 381—386

The autonomic mechanisms controlling cardio- ascular adjustments to diving have been stu lied in ducks using drugs which block adrenergic α receptors (pl ntolam ne) or d [illegible] te catechol amine stores (reserpine) In an mals thus treated there was a marked change in the cardio-vascular reflex pattern upon diving The vasoconstrictor response regularly seen in normal submerged animals appeared to be abolished and the d ving brad cardia was only poorly developed As a consequence the treated animals had lost their capability to endure subm rsion for prolonged periods of time the catecholamine depl ter being more effective in this respect than the α receptor blocking agent However if noradrenal ne was administered to a submerged animal pre treated with reserpine peripheral vasoconstriction and bradycardia took pla e momentarily It is concluded that the initial quick slowi g of the heart whi h takes place upon submersion results from an immediate parasympatl etic act ity as ociat d with the reflex apnoea The profound diving bradycardia gradually developing may be due to baro-recepto stimulation related to the periph eral vasoconstriction

The nervous mechanisms which control the cardio-vascular responses exhibited by ducks during diving have received much attention during the last decade

Several workers have made use of pharmacological tools in order to study separate l the contributions of the sympathetic and the parasympathetic divisions of the autonomic nervous s tem Thus the original observation of Richet (1894) that the diving bradycardia in ducks is abolished by atropine has been reconfirmed in several recent inve tigations (Folkow Nilsson and Yonce 1967 Butler and Jones 1968 Butler and Jones 1971) Inquiries into the sympathetic involvement have been con ducted using drugs which interfere with the adrenergic mechanisms in different [illegible] With respect to adrenergic α receptor blockade in particular it has been [illegible] shown that phenoxybenzamine abolishes the systemic vasoconstriction [illegible] normally elicited upon submersion (Butler and Jones 1971 Blix Gaut [illegible] Refsum in press) Moreover Kobinger and Oda (1969) tried to inve [illegible] effect of catecholamine depletion through pre treatment of their animal

pine However they reported that their ducks did not respond very well to this treatment but appeared agitated while submerged and that they usually died during the experiment

The investigation reported in this paper serves a two fold purpose First, we wished to compare the effects of adrenergic α receptor blockade and catecholamine depletion with respect to the diving endurance of ducks Second we have tried to separate the sympathetic and the parasympathetic contributions to the early slowing of the heart evoked in submerged ducks The results obtained have enabled us to suggest a possible explanation for the gradual slowing of the heart rate towards the extreme level of bradycardia which becomes established later in the dive

## Material and Methods

*Eight ducks of both sexes weighing 2—3 kg were used They were normally able to endure* forced submersion for more than 10 min In preparation for an experiment intravascular catheters were inserted into an artery and a vein of the wing The tip of the latter catheter *was placed in a position near the right atrium Intravascular pressures were recorded using* Statham transducers (P 23 Dc and P 23 BB) connected to a Beckman RS Polygraph

Blocking of the adrenergic α receptors was carried out by i v administration of phentolamine *(Regitin® Ciba) The dose was adjusted so as to give maximal effect in our experiments* 3 mg/kg b wt

In other experiments catecholamine depletion was caused by i m administration of reserpine *(Serpasil® Ciba) in doses of 0 5—1 mg every 12 h for 48—60 h the total dose being dependent* upon the response of the individual animal The last injection was given approximately 4 h prior to the experiment Finally noradrenaline was injected i v during diving in some of the *animals pretreated with reserpine Ampullas containing 1 mg/ml were diluted into 9 ml of a* physiological saline solution (0 9 %) and administered i v over 15—20 s

## Results

*Normal cardio vascular responses* to a 10 min submersion of the head of an intact duck are shown in Fig 1 A This illustration includes the first and the final 30 s periods of the dive together with the end of the prediving and the beginning of the recovery periods

The heart rate slowed down gradually during the initial 45 s from a resting rate of 175 beats/min to 15 beats/min This level of bradycardia persisted throughout the dive The mean arterial blood pressure showed a transient rise upon descent from a resting value of approximately 125 mm Hg to 150 mm Hg It then fell gradually to about 100 mm Hg as the bradycardia became established An immediate pressor response took place upon emersion when the mean arterial pressure rose to 200 mm Hg with a subsequent fall to the resting level Simultaneously the heart rate increased to 360 beats/min

The central venous pressure rose momentarily upon submersion *in a stepwise* fashion from about 5 to roughly 12 cm $H_2O$ and it continued to *increase throughout* the underwater episode to more than 20 cm $H_2O$

*Alpha adrenergic blockade* was carried out using phentolamine The results obtained are presented in Fig 1 B The total diving performance lasted for 150 s The animal appeared to experience serious difficulties in enduring any further period of submergence The figure includes the start and the end of the dive and a part of

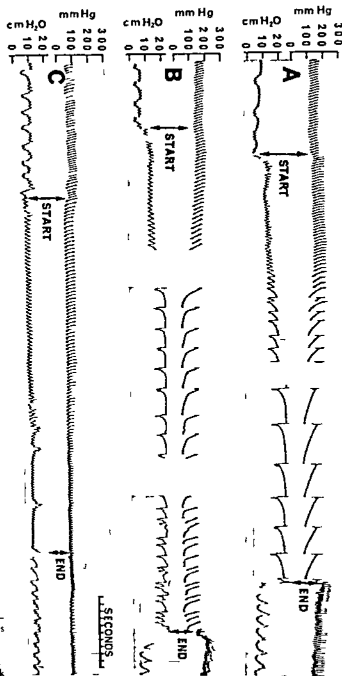



Fig 1 Cardiovascular adjustments of ducks to submersion Each panel shows arterial pressure (upper tracing) and central venous pressure (lower tracing) A Responses of an intact duck at the beginning and the end of a dive lasting fro 10 min B Responses of a duck where the adrenergic α receptors had been blocked by means of phentolamine 3 mg/kg b wt Records from the beginning from the middle and from the end of a dive lasting for approximately 150 s C Responses of a duck pretreated with reserpine 1 mg every 12 h for 60 h Registration before during and after diving




Fig 2 Heart rate and arterial blood pressure before during and after "diving of a duck pre treated with reserpine and with noradrenaline administered i v during the submersion

the record taken after approximately 75 s of diving The reflex adjustments to water immersion of the head evolved in much the same way as already described for the untreated animal Thus although phentolamine has a cardioacceleratory effect of its own this did not prevent the development of a diving bradycardia However, it is obvious from a study of the diastolic limb of the pressure curve after 75 s of diving that the blood ejected from the heart into the large arteries is not being slowly and evenly drained into a periphery with marked vasoconstriction as is the case under normal conditions There is now a much more steep initial fall of the curve in early diastole with a subsequent leveling out towards the end-diastolic period This pattern suggests that the animal is left unable to constrict his vascular beds which are normally more or less shut off from the circulation during diving Moreover the central venous pressure did not usually rise to the normal maximal level indicating tht the central displacement of the circulating blood is less pronounced with the adrenergic $\alpha$ receptors blocked

After approximately 2 min of diving cardio-acceleration with arrhythmia followed and the record taken after 120 s confirmed the subjective impression of imminent circulatory collapse

*Catecholamine depletion* was carried out in 3 ducks who underwent 2 expts each The record obtained in one of them is shown in Fig 1 C The starting conditions is of course very different from those illustrated in Fig 1 A and B The mean arterial pressure was as low as approximately 80 mm Hg More striking however is the fact that the moderate cardiac retardation which took place immediately upon descent into water remained Thus the heart rate fell abruptly from 180 to 120 beats/min but with no further gradual reduction Rather cardio-acelleration took place after approximately 30 s of submersion and towards the end of the underwater episode which lasted for roughly 45 s the heart frequency had reached the pre-diving rate There were no obvious changes in the intravascular pressures recorded during submersion which probably lasted for as long a period of time as this duck was able to endure Moreover the striking tachycardia and the rise in systemic blood pressure normally displayed upon completion of a dive were absent

Two of the ducks pre treated with reserpine received non adrenalin i v during diving An example of such an experiment is shown in Fig 2 Immersion of the head into water momentarily evoked some bradycardia The heart rate slowed abruptly from a prediving value of 180 beats/min to 120 beats/min and the diastolic

and the mean arterial blood pressure fell imediately No further cardiac retardation took place during the initial 20 s of this dive When nor adrenalin was administered the systemic blood pressure rose instantaneously with a concomitant development of bradycardia to a steady rate of 48 beats/min

## Discussion

Ducks pharmacologically deprived of the peripheral vasoconstrictor response which normally takes place upon submersion lose their capability to dive for prolonged periods of time Thus adrenergic $\alpha$ receptor blockade with large doses of phentolamine reduced their diving capacity from more than 10 to approximately 3 min in our experiments Catecholamine depletion resulting from treatment with reserpine made even dives of less than 1 min duration difficult to endure Impaired sympathetic vasoconstrictor function during submergence became evident in two different ways First the slow drop in arterial pressure recorded during cardiac diastole typically a nearly linear function of time (Fig 1 A) underwent a marked change when phentolamine was injected the pressure now fell abruptly in early diastole with a protracted low pressure interval supervening (Fig 1 B) Catecholamine depletion abolished the arterial pressure response to submersion althogether (Fig 1 C) Second the central venous pressure elevation consistently observed in submerged intact animals (Fig 1 A) became less prominent when the adrenergic $\alpha$ receptors had been blocked (Fig 1 B) With catecholamines depleted this response disappeared entirely (Fig 1 C) Our results therefore indicate that peripheral vasoconstriction is an essential physiological event in the development of the oxygen conserving cardiovascular adjustments to diving

Reserpine induced a clear dissociation of the automatic mechanisms normally operating in concert during a dive This finding together with the circulatory responses seen subsequent to administration of noradrenaline reveals certain discrete contributions from the sympathetic and the parasympathetic nervous system Thus the immediate but relatively inconspicuous drop in heart rate consistently observed upon submersion in ducks pretreated with reserpine (Fig 1 C and 2) may be due to a vagal discharge This effect appears to be closely integrated with the nervous mechanisms which stop the respiratory movements instantaneously when the nares become submerged We have tentatively interpreted this initial bradycardia as being part of the trigeminal reflex described by Andersen (1963) Without peripheral vasoconstriction ensuing no further bradycardia took place However when noradrenaline was administered a conspicuous diving bradycardia was established from one beat of the heart to the next The arterial blood pressure increased simultaneously as is sometimes observed in ducks in the very beginning of the dive (*cf* Andersen 1966) Our results therefore upport the hypothesis that the marked diving bradycardia typical of intact ducks develops gradually in accord with baroreceptor stimulation following the increase in peripheral resistance In this way the cardiovascular controlling mechanism apparently exert a beat to-beat adjustment of heart rate and

total peripheral resistance in such a way that the stroke volume and the arterial blood pressure remains largely unchanged during the under water episode Moreover it appears unlikely from the data presented in this paper that there is any chemoreceptor influence on the pattern of cardiovascular adjustments in the beginning of a dive

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Acta physiol scand 1974 90 387—400
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# The Effects of Repetitive Stimulation on the Action Potential and the Twitch of Rat Muscle

By

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## Abstract

HANSON J *The effects of repetitive stimulation on the action potential and the twitch of rat muscle* Acta physiol scand 1974 *90* 387—400

Membrane potentials and contraction curves of different types of rat muscle fibre have been studied at body and room temperatures with special regard to the effects of repetitive stimulation Fast fibres (from the surface of the extensor muscles of the antebrachium) have a higher resting membrane potential a higher amplitude and shorter duration of the spike of the action potential and a larger early negative after potential than soleus fibres In the fast fibres stimulation at 10 Hz for 30 s produced a decrease of the resting membrane potential a decrease in the amplitude of the spike of the action potential and an increase in its duration as well as an increase in the early negative after potential In soleus fibres such changes were rare These findings indicate that there are differences in the membrane properties between the two types of muscle fibres in addition to previously known differences in mechanical properties Repetitive stimulation of fast fibres at body temperature caused a rapid increase in the twitch amplitude (staircase phenomenon) This increase occurred before any appreciable change in the action potential Stimulation at room temperature caused only small changes in the twitch amplitude but marked increase in the twitch duration This suggests that repetitive stimulation may alter both the amplitude and the duration of the active state curve

In a previous investigation the effetcs of repetitive stimulation were studied in isolated frog muscle (Hanson and Persson 1971) Characteristic changes in the membrane potentials were found mainly an increase in the duration of the spike and in the amplitude of the early negative after potential These changes had no causal relationship to changes in the muscle twitch

Since earlier contraction studies showed that mammalian muscle fibres of different types behave differently when subjected to repetitive stimulation (Ranvier 1880 Brown and Euler 1938 del Pozo 1942 Bowman *et al* 1962 Edström and Kugelberg 1968 Nyström 1968 Burke *et al* 1971 Kugelberg 1971 Tsairis *et al* 1971) the present study was undertaken to determine whether the effects of repetitive stimulation on the membrane potentials also vary from one type of muscle fibre to another and furthermore whether the effects on the membrane potentials are correlated with the potentiation or with the fatigue of the twitch caused by the repetitive stimulation

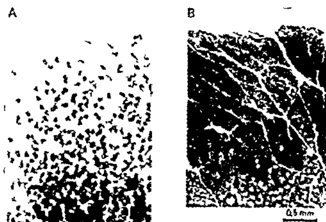



Fig 2 Sections of the extensor muscles of the antebrachium of rat stained for SDH (A) and myofibrillar ATPase (B) Surface of preparation at top

TABLE I Results of intracellular recordings from surface fibres of the extensor muscles of the antebrachium and the soleus muscle at 37–38 C The values are the means ± S D The differences between the muscles are significant ($p < 0.001$) The resting membrane potentials ($E_R$) of the soleus fibres are not normally distributed (comparatively many values near 70 mV)

| | Extensor muscles surface fibres n = 150 (16 muscles) | Soleus fibres n = 61 (5 muscles) |
|---|---|---|
| $E_R$ mV | 77.1 ±3.9 | 73.7 ±2.8 |
| $V_s$ mV | 102.9 ±6.8 | 93.8 ±6.2 |
| Dur ms | 0.62±0.08 | 0.84±0.07 |
| $V_A$ mV | 7.2 ±2.2 | 5.1 ±2.2 |
| $E_A$ mV | 69.9 ±3.0 | 68.6 ±2.1 |

TABLE II Twitch data of preparations from the surface and the deep parts of the extensor muscles of the antebrachium and from the soleus muscle Temperature 37—38 C The values are the means ± S D

| | *Extensor muscles* preparations from the surface n = 9 | *Extensor muscles* preparations from the deep parts n = 6 | Soleus muscle preparations n = 8 |
|---|---|---|---|
| *Contraction time* ms | 7.1±0.7 | 9.8±0.7 | 26.6±2.6 |
| Half relaxation time ms | 7.3±1.1 | 15.7±2.8 | 37.5±6.5 |

soleus muscle fibres in that the former had a higher resting membrane potential a higher amplitude and a shorter duration of the spike of the action potential and also a higher amplitude of the early negative after potential ($V_A$) The absolute value of the post spike membrane potential ($E_A$) was higher in the extensor fibres than in the soleus fibres The differences were statistically significant ($p < 0.001$) although the range of values overlapped

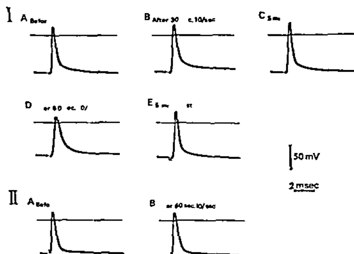


Fig 3 I Action potentials at 37—38 C from a surface fibre of extensor muscles of the antebrachium, A before and B immediately after stimulation at 10 Hz for 30 s C after 5 min rest D immediately after stimulation at 10 Hz for 60 s E after 5 min rest. II Action potentials at the same temperature from a surface fibre of the soleus muscle A before and B immediately after stimulation at 10 Hz for 60 s Level of zero potential indicated by horizontal line

TABLE III Results of intracellular recordings at 37—38 C from surface fibres of the extensor muscles of the antebrachium before and after repetitive stimulation All recordings are from different fibres The values are the means ± S D All differences are significant ($p < 0\ 001$) The values of the duration after repetitive stimulation are not normally distributed (slightly bimodal distribution curve)

| | Before rep stim n=150 | After 10 Hz f 30 s n=39 |
|---|---|---|
| $E_R$ mV | 77 1 ± 3 9 | 73 2 ± 2 9 |
| $V_S$ mV | 102 9 ± 6 8 | 97 8 ± 6 6 |
| Dur ms | 0 62 ± 0 08 | 0 9 ± 0 13 |
| $V_A$ mV | 7 2 ± 2 2 | 9 7 ± 2 7 |
| $E_A$ mV | 69 9 ± 3 0 | 63 5 ± 2 9 |

*Mechanical properties of previously unstimulated preparations*

The contraction times and the half relaxation times (*cf* Fig 2 B) are shown in Table II The twitch was considerably slower in soleus than in extensor fibres Bundles of fibres from the surface of the extensors were somewhat faster than those from the deep parts of the same muscles All differences were significant ($p < 0\ 001$)

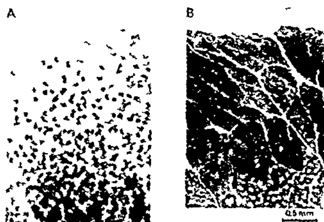



Fig 2 Sections of the extensor muscles of the antebrachium of rat stained for SDH (A) and myofibrillar ATPase (B) Surface of preparation at top

TABLE I Results of intracellular recordings from surface fibres of the extensor muscles of the antebrachium and the soleus muscle at 37–38 C. The values are the means ± S D The differences between the muscles are significant ($p < 0\ 001$) The resting membrane potentials ($E_R$) of the soleus fibres are not normally distributed (comparatively many values near 70 mV)

| | Extensor muscles surface fibres n = 150 (16 muscles) | Soleus fibres n = 61 (5 muscles) |
|---|---|---|
| $E_R$ mV | 77 1 ± 3 9 | 73 7 ± 2 8 |
| $V_S$ mV | 102 9 ± 6 8 | 93 8 ± 6 2 |
| Dur ms | 0 62 ± 0 08 | 0 84 ± 0 07 |
| $V_A$ mV | 7 2 ± 2 2 | 5 1 ± 2 2 |
| $E_N$ mV | 69 9 ± 3 0 | 68 6 ± 2 1 |

TABLE II Twitch data of preparations from the surface and the deep parts of the extensor muscle of the antebrachium and from the soleus muscle Temperature 37—38 C The values are the means ± S D

| | Extensor muscles preparations from the surface n = 9 | Extensor muscles preparations from the deep parts n = 6 | Soleus muscle preparations n = 8 |
|---|---|---|---|
| Contraction time ms | 7 1 ± 0 7 | 9 8 ± 0 7 | 26 6 ± 9 6 |
| *Half* relaxation time ms | 7 3 ± 1 1 | 15 7 ± 2 8 | 37 5 ± 6 5 |

soleus muscle fibres in that the former had a higher resting membrane potential a higher amplitude and a shorter duration of the spike of the action potential and also a higher amplitude of the early negative after potential ($V_A$) The absolute value of the post spike membrane potential ($E_N$) was higher in the extensor fibres than in the soleus fibres The differences were statistically significant ($p < 0\ 001$) although the range of values overlapped

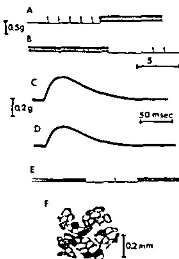



Fig 6

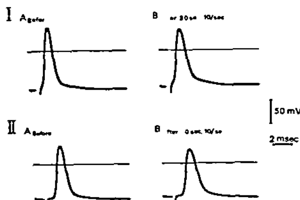



Fig 7

Fig 6 Contraction records at 37—38 C of a preparation of soleus fibres A and B are tension recordings at the beginning and end of repetitive stimulation (8 Hz sequence of 60 s duration) C and D are contraction curves before and 30 s after this stimulation period E is a recording after 1 h stimulation at 8 Hz F is a section of the preparation stained for SDH

Fig 7 I Action potentials at 21 5—22 5 C from a surface fibre of the extensor muscles of the antebrachium A before B immediately after stimulation at 10 Hz for 30 s II Action potentials at the same temperature from a surface fibre of the soleus muscle A before B immediately after stimulation at 10 Hz for 60 s Level of zero potential indicated by horizontal line

Stimulation for 30 s caused more marked changes (Fig 3 I A—C Table III) There was a decrease in the resting membrane potential a decrease in the amplitude of the spike of the action potential an increase in its duration and a more pronounced decrease in the post spike membrane potential ($E_A$) The changes were statistically significant ($p < 0.001$) They subsided within 5 min rest

After 60 s stimulation the changes were still more pronounced (Fig 3 ID)

In soleus fibres the only change seen after 60 s stimulation at 10 Hz was a small increase in the resting membrane potential in about half of the fibres examined (Fig 3 II A II B) Statistical analysis of data obtained from 18 soleus fibres stimulated for 60 s at 10 Hz showed no significant difference from previously unstimulated fibres ($p > 0.05$) The fibres could be stimulated for longer periods with still very small effects on the membrane potentials After 5 min stimulation at 10 Hz sometimes however changes of the same type as those seen in the extensor fibres were seen also in soleus fibres They subsided within about 1 min

No repetitive discharges were seen either in the soleus or in the extensor muscle preparations

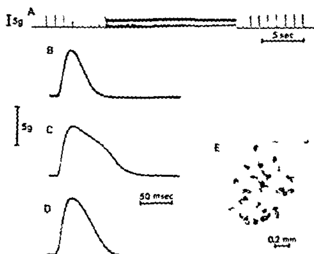



Fig 8 Contraction records at 21.5—22.5 C of a preparation from the surface of the extensors muscles of the antebrachium consisting mostly of fibres poor in SDH (E) A is a continuous recording of the tension during repetitive stimulation (10 Hz sequence of 15 s duration) B is a contraction curve before the stimulation period C and D are contraction curves 30 s and 2 min after

*Effects of repetitive stimulation on the twitch*

*Preparations from the surface of the extensors* The initial stimulation at 1 Hz produced no change in the twitch Stimulation at 10 Hz produced a rapid increase in the amplitude of the twitch (staircase phenomenon Treppe Fig 4), which reached its maximum after about 15 s stimulation (mean 257 % of the initial peak tension) When the stimulation frequency after 15 s stimulation at 10 Hz was reduced to 1 Hz there was a further slight increase in the twitch amplitude Neither the contraction time nor the half relaxation time was significantly influenced ($p > 0.05$) After the end of the repetitive stimulation the twitch amplitude slowly decreased to the original amplitude or somewhat less after 5 min rest

The staircase phenomenon was also seen at a stimulus frequency of 3—4 Hz but was less pronounced than at 10 Hz In preparations stimulated repetitively over several periods the staircase phenomenon was less pronounced during the later periods

*Preparations from the deep parts of the extensors* There was a successive increase in the twitch amplitude during stimulation at 10 Hz (Fig 5) However the increase was generally less than in surface preparations (mean amplitude after 15 s stimulation was 133 % of the initial amplitude) There was no significant change in contraction time ($p > 0.05$) Half relaxation time decreased slightly ($p < 0.05$)

*Soleus muscle preparations* A lower stimulus frequency (8 Hz) was used in these preparations in order to avoid the strong fusion of twitches which occurs with higher frequencies In about one half of the preparations there was during the first few seconds of stimulation a small rapid increase in the twitch amplitude (Fig 6 A) which occasionally rose to 125 % of the initial amplitude With longer periods of stimulation the contraction curve for all soleus muscle preparations showed a slow progressive decrease in amplitude (Fig 6 B C D) After 60 s stimulation at 8 Hz there was no significant change in contraction time or half relaxation time ($p > 0.05$)

TABLE IV Twitch data of preparations from the surface and from the deep parts of the extensor muscles of the antebrachium and from the soleus muscle Temperature 21 5—22 5 C The values are the means±S D

| | Extensor muscles preparations from the surface n=8 | Extensor muscles preparations from the deep parts n=10 | Soleus muscle preparat ons n=9 |
|---|---|---|---|
| Contraction time ms | 22 8±1 5 | 28 2±3 3 | 65 3±5 1 |
| Half relaxation time ms | 23 0±3 4 | 38 7±11 6 | 96 2±6 6 |

Soleus muscle preparations tolerated prolonged stimulation considerably better than extensor muscle preparations Even after 1 h stimulation the decrease in twitch tension was often moderate (Fig 6 E)

B *Experiments at 21.5—22 5° C*

*Electrophysiological properties of previously unstimulated preparations*

The resting membrane potentials at room temperature were about the same as at body temperature The duration of the spike was on the average almost 3 times as long as at body temperature

The rate of decline of the negative after potential was about twice as fast at room temperature as at body temperature This often made it difficult to distinguish the negative after potential from the spike of the action potential and it was not possible to measure its amplitude accurately However the after potential was more prominent in extensor fibers (Fig 7 I A) than in soleus fibres (Fig 7 II A)

*Mechanical properties of previously unstimulated preparations*

The means of the contraction times and half relaxation times are shown in Table IV These values are about 2—3 times longer than those obtained at body temperature As at body temperature the twitch was much slower in soleus than in extensor preparations ($p < 0.001$) There was also a difference between the 2 kinds of extensor muscle preparations the twitch being slower in those from the deep parts than in those from the surface layers ($p < 0.01$) Extensor muscle preparations studied both at room and body temperature showed a considerably higher twitch peak tension at room temperature than at body temperature The soleus muscle preparations showed about the same tension

The exten or surface preparations used in the e experiments contained on the average 77 % fibres with low SDH activity The preparations from the deep parts of the extensors contained on the average 48 % fibres with intermediate SDH activity and 48 % fibres with high SDH activity The soleus muscle preparations contained on the average about 85 % fibres with intermediate SDH activity

As at body temperature there was thus no correlation between the SDH activity and the time course of the twitch

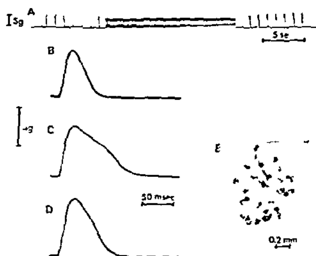



Fig 8 Contraction records at 21 5—29 5 C of a preparation from the surface of the extensors muscles of the antebrachium consisting mostly of fibres poor in SDH (E) A is a continuous recording of the tension during repetitive stimulation (10 Hz sequence of 15 s duration) B is a contraction curve before the stimulation period C and D are contraction curves 30 s and 2 min after

*Effects of repetitive stimulation on the twitch*

*Preparations from the surface of the extensors* The initial stimulation at 1 Hz produced no change in the twitch Stimulation at 10 Hz produced a rapid increase in the amplitude of the twitch (staircase phenomenon Treppe, Fig 4) which reached its maximum after about 15 s stimulation (mean 257 % of the initial peak tension) When the stimulation frequency after 15 s stimulation at 10 Hz was reduced to 1 Hz there was a further slight increase in the twitch amplitude Neither the contraction time nor the half relaxation time was significantly influenced ($p > 0\ 05$) After the end of the repetitive stimulation the twitch amplitude slowly decreased to the original amplitude or somewhat less after 5 min rest

The staircase phenomenon was also seen at a stimulus frequency of 3—4 Hz but was less pronounced than at 10 Hz In preparations stimulated repetitively over several periods the staircase phenomenon was less pronounced during the later periods

*Preparations from the deep parts of the extensors* There was a successive increase in the twitch amplitude during stimulation at 10 Hz (Fig 5) However the increase was generally less than in surface preparations (mean amplitude after 15 s stimulation was 133 % of the initial amplitude) There was no significant change in contraction time ($p > 0\ 05$) Half relaxation time decreased slightly ($p < 0\ 05$)

*Soleus muscle preparations* A lower stimulus frequency (8 Hz) was used in these preparations in order to avoid the strong fusion of twitches which occurs with higher frequencies In about one half of the preparations there was during the first few seconds of stimulation a small rapid increase in the twitch amplitude (Fig 6 A) which occasionally rose to 125 % of the initial amplitude With longer periods of stimulation the contraction curve for all soleus muscle preparations showed a slow progressive decrease in amplitude (Fig 6 B C D) After 60 s stimulation at 8 Hz there was no significant change in contraction time or half relaxation time ($p > 0\ 05$)

Sreter and Woo 1963 Yonemura 1967 Hoh and Salafsky 1971) and the higher intracellular Na concentration (Sreter and Woo 1963 Yonemura 1967) in soleus fibres than in fast muscle fibres However a full explanation of the differences requires knowledge of the ion permeabilities of the membrane at rest and during an action potential

The appearance of the early negative after potential is dependent on the transverse tubular system (Gage and Eisenberg 1967 1969 Hanson and Persson 1971) and it seems highly probable that the differences in the amplitude of the after potential between fast and slow fibres is due to difference in the size of the transverse tubular system Electron microscopic investigations have in fact shown that the transverse tubular system is more extensive in fast than in slow muscles of the mouse (Duchen 1971 Luff and Atwood 1971)

The after potential in both the rat muscles studied here is considerably smaller than in the frog muscles studied earlier (Persson 1963 Hanson and Persson 1971) This can only partly be explained by the small differences in the transverse tubular system which may exist to judge from electron microscopic investigations (Huxley 1964 Peachey 1965 Eisenberg and Gage 1969 Luff and Atwood 1971) The greater part of the difference must be due to differences in the membrane characteristics of frog and rat muscle fibres

The contraction studies showed as was expected that the extensor muscle fibres are considerably faster than soleus fibres According to several investigators this difference appears to be related to differences in the intensity of myofibrillar ATPase activity in the fibres and not to the SDH activity (Barany 1967 Edström and Kugelberg 1968 Burke *et al* 1971 Edgerton and Simpson 1971 Kugelberg 1971 Tsairis *et al* 1971 Close 1972) This is consistent with the present findings

The small difference in the time course of the twitch found between the two kinds of extensor muscle preparations studied in the present investigation would appear to be due to differences in the occurrence of a few fibres with low ATPase activity

Repetitive stimulation of the extensor muscle preparation (especially the surface fibres) at body temperature caused a rapid increase in the twitch amplitude while stimulation of the soleus muscle preparations caused little change This is in accordance with earlier investigations (Ranvier 1880 Brown and Euler 1938 Feng 1939 del Pozo 1942 Bowman *et al* 1962 Standaert 1964 Nystrom 1968)

Repetitive stimulation caused changes in the intracellularly recorded potentials of the extensor surface fibres (decrease of the resting membrane potential and spike amplitude increase of spike duration and early negative after potential and also decrease of the post spike membrane potential F ) These effects of repetitive stimulation are of the same kind as those seen in frog muscle fibres (Etzensperger 1961 b Persson 1963 Hanson and Persson 1971) but less pronounced In soleus fibres there were only very small changes of the intracellular potentials

It has been assumed that repetitive stimulation increases the extracellular K concentration which is said to cause the increase of the twitch (Brown and Euler 1938

Waler 1948 Etzensperger 1961 a 1961 b) Such increase in the K concentration would be associated with changes in the membrane potentials Chemical potentiation of the twitch has also been attributed to an increase in the duration of the spike of the action potential by some authors (Sandow 1964 Sandow *et al* 1965 Edman *et al* 1966 Taylor *et al* 1972) and to an increase in the amplitude of the early negative after potential by others (Etzensperger *et al* 1956 Etzensperger 1957 Lubin 1957)

These hypotheses may appear to be supported by the present finding that long time repetitive stimulation of surface extensor muscle preparations produced changes in both the twitch and the membrane potentials while repetitive stimulation of soleus fibres produced little or no change either in the twitch or in the membrane potentials However this explanation of the potentiation is less probable since stimulation at 10 Hz for 15 s produced a very marked potentiation of the twitch while the changes in the membrane potentials were very small (the negative after potential) or absent (resting membrane potential spike amplitude and duration) The mechanism underlying the potentiation caused by repetitive stimulation must be found in a link later than the action potential in the chain of events leading to the contraction It seems plausible that the insignificant changes both in the twitch and in the intracellularly recorded potentials during repetitive stimulation of soleus fibres are due to the high endurance capacity of these fibres

The increase in the twitch seen following reduction of stimulation frequency after the 15 s stimulation period is probably a recovery due to a redistribution of the intracellular calcium (Connolly *et al* 1971) It is possible that changes in the calcium turnover might also explain facilitation of the twitch caused by repetitive stimulation (Chapman 1971)

Repetitive stimulation at body temperature had no effect on the duration of the twitch This indicates that the increase in the twitch amplitude is probably due to an increase in the intensity of the active state of the contractile elements (Close and Hoh 1968 b Desmedt and Hainaut 1968 *cf* Rosenfalck 1968)

The amplitude of the twitch of the extensor muscle preparations was considerably higher at room temperature than at body temperature During repetitive stimulation there was no further increase but instead an initial decrease However there was a marked increase in the duration of the twitch mainly in the relaxation phase (*cf* Close and Hoh 1968 a) To judge from the change in the twitch repetitive stimulation at room temperature apparently produces an increase in the duration of the active state

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# Labelled 5-Hydroxytryptamine and 5-Hydroxyindoleacetic Acid Formed in Vivo from ³H-Tryptophan in Rat Brain, Effect of Probenecid*)

By

JOHAN SCHUBERT

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## Abstract

SCHUBERT J *Labelled 5 hydroxytryptamine and 5 hydroxyindoleacetic acid formed in vivo from ³H tryptophan in rat brain effect of probenecid* Acta physiol scand 1974 *90* 401—408

Procedures were developed for determination of labelled 5 hydroxytryptamine (5 HT) and 5-hydroxyindoleacetic acid (5-HIAA) formed *in vivo* from ³H tryptophan in rat brain Tryptophan 5 HT and 5 HIAA in brain extracts were isolated on columns of Dowex 50W X4 Amberlite CG 50 and Sephadex G 10 recoveries being 64 % 78 % and 66 respectively Following i v injection of ³H tryptophan labelled 5 HT and 5 HIAA accumulated in brain reaching maximal levels after approximately 15 and 60 min respectively The relationship between the specific activity curves for 5 HT and 5 HIAA appeared to be consistent with single compartment kinetics for 5 HT The effect of probenecid (200 mg/kg) on the amounts of labelled 5-HT and 5 HIAA formed from ³H tryptophan indicates that besides inhibiting 5 HIAA efflux from brain the drug also enhances 5 HT synthesis


Estimation of the rate of 5 hydroxytryptamine (5 HT) turnover in rat brain can be obtained by determination of accumulation or disappearance rates for labelled 5 HT formed *in vivo* from radioactively labelled tryptophan (Thierry Fekete and Glowinski 1968 Lin *et al* 1969 Neff *et al* 1971 Schubert and Sedvall 1972) Such a procedure is valid only during steady state conditions for the endogenous amine and its precursor In experiments where the level of 5 HT is changing and synthesis of 5 HT does not equal its catabolism determination of the rate of accumulation of 5 hydroxyindoleacetic acid (5 HIAA) formed from ³H tryptophan should give complementary information regarding changes in 5 HT metabolism

For this purpose methods for determination of labelled 5 HT and 5 HIAA formed *in vivo* from ³H tryptophan in br in have been developed Isolation of the com

* A preliminary report of part of this t dy was presented at the Fifth International Congress on Pharmacology in San F anci co July 1 2

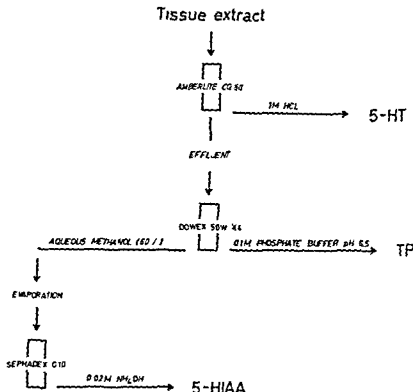



Fig 1 Schematic illustration of column procedures for isolation of tryptophan (TP) 5 HT and 5 HIAA from brain extracts

pounds involves ion exchange chromatography and gel filtration based on modifications of the procedures described by Jonsson and Lewander (1970) and Lindqvist (1971) The time courses for changes of specific activity of tryptophan 5 HT and 5 HIAA in brain after intravenous injection of $^3H$ tryptophan to rats are described. The procedure was used to study effects of probenecid

## Materials and methods

Male Sprague Dawley rats weighing 150—170 g were used $^3H$ 1 Tryptophan (5 1)—5 30 Ci mmole generally labelled 5-hydroxytryptamine 3 $^{14}C$ creatinine sulphate 40 Ci/mmole) and 5-hydroxyindoleacetic-carboxyl $^{14}C$-acid (6 5 mCi/mmole) were obtained from New England Nuclear Corp $^3H$ Tryptophan was stored in 50 % aqueous ethanol which was evaporated before use When administered i v $^3H$ tryptophan was dissolved in 0 9 % saline and 0 5 ml was injected into a tail vein Probenecid (Astra Sodertalje Sweden) was dissolved in 0 9 % saline and injected intraperitoneally in a volume of 0 5 ml

*Extraction of indoles from brain* Animals were stunned and decapitated and brains were removed rapidly cooled on ice and weighed *Homogenization of the brains was performed in* 8 ml of ice-cold 0 4 M perchloric acid containing 0 02 % ascorbic acid The homogenates were centrifuged at 3 000 x g in 0 C for 30 min After centrifugation an aliquot of the supernatant was taken for determination of total radioactivity The pH of the supernatant was adjusted to 6 5 with N $K_2CO_3$ Precipitated potassium perchlorate was removed by centrifugation

TABLE I Recovery of labelled tryptophan 5-HT and 5 HIAA after chromatographic isolations The amounts of radioactivity in Amberlite Dowex and Sephadex eluates are expressed as percentage ± S E of added amounts (2 10 dpm) in 4 brain extracts

| | H Tryptophan | H 5 HT | $^3$H 5 HIAA |
|---|---|---|---|
| Amberlite eluate | < 0 1 | 78 ± 1 | < 0 1 |
| Dowex eluate | 64 ± 1 | 0 6 ± 0 1 | 4 3 ± 0 1 |
| Sephadex eluate | < 0 1 | < 0 1 | 66 ± 1 |

*Isolation of tryptophan 5 HT and 5 HIAA from brain extracts* The tissue extracts were applied on columns of Amberlite CG 50 type 1 (7 0 × 30 mm K form) (Fig 1) Ten ml of water was washed through the columns into the effluent. The columns were then rinsed with 50 ml of water 50 ml of boiling water followed by 0 5 ml of 1 M HCl Elution of 5 HT absorbed to the columns was performed with 5 0 ml of 1 M HCl (Amberlite eluate) Ascorbic acid (0 02 %) was added to all aliquots throughout the procedure to prevent oxidation of 5 HT The recovery of labelled 5-HT was 78 ± 1 % (mean ± S E ) (Table I) The pH of the Amberlite effluent was adjusted to 2 0 with HCl and the solution was poured onto a column of Dowex 50W X4 (7 0 × 35 mm K form) Elution of 5 HIAA was performed with 10 ml of 60 % aqueous methanol The column was washed with 3 ml of 0 1 M phosphate buffer pH 6 5 Tryptophan was eluted with another 15 ml of the phosphate buffer (Dowex eluate), the recovery being 64 ± 1 % (Table I) Isolation of tritium labelled and unlabelled tryptophan yielded similar recoveries demonstrating that no tritium loss occurred during the chromatographic purification (unpublished results) In order to purify the methanol eluate from remaining tryptophan (2—4 %) it was evaporated under reduced pressure in a flash evaporator at 40 C The residue was dissolved in water pH adjusted to 2 0 and applied on a column of Sephadex G 10 (7 0 × 30 mm) The column was washed with 2 × 5 ml of 0 1 M HCl (containing 0 02 % ascorbic acid) Elution of 5 HIAA was performed with 4 ml of 0 02 M $NH_4OH$ (Sephadex eluate) without the presence of measureable amounts of tryptophan or 5 HT (Table 1)

*Determination of labelled indoles* Radioactivity was determined by dissolving 1 ml of the Amberlite Dowex and Sephadex eluates respectively in Insta Gel (Packard Instr Co ) followed by liquid scintillation counting in a Packard Tri Carb spectrometer with an absolute activity analyzer For calculation of the amount of labelled indoles present in the eluates correction for tritium loss during the biotransformation of $^3$H tryptophan to $^3$H 5 HIAA was performed It was assumed that the $^3$H tryptophan was uniformly labelled (Neff *et al* 1971) and that tritium associated with the carboxyl and amine group as well as with the indole nitrogen was exchanged for hydrogen in the aqueous solution During the conversion of $^3$H tryptophan to $^3$H 5 HT and of $^3$H 5 HT to $^3$H 5 HIAA a loss of one tritium atom at each step was reckoned with The amount of labelled indoles present in the eluates were corrected for recoveries (Table I)

*Identification of labelled indoles in brain* Paper chromatographic evidence of the presence of labelled 5 HT in the Amberlite eluate and labelled tryptophan in the Dowex eluate has earlier been presented (Schubert Nyback and Sedvall 1970 Schubert and Sedvall 197?)

For the identification of labelled 5 HIAA formed *in vivo* from $^3$H tryptophan in brain the Sephadex eluate was neutralized and evaporated almost to dryness The residue was applied as a strip on Whatman No 1 filter paper together with reference compounds The chromatograms were processed by ascending chromatography in ? solvent systems After drying the chromatograms the reference spots were visualized in UV light or by spraying the chromatograms with Ehrlich reagent (Jepson 1960) For localization of radioactive peaks the chromatograms were cut in 1 cm strips which were eluated with 3 ml of 1 M HCl and dissolved in Insta Gel Radioactivity was counted in vials by liquid scintillation spectrometry The paper chromatographies of the Sephadex eluate revealed distinct peaks which had the same chromatographic mobility as authentic 5 HIAA (Fig 2) Significant amounts of other labelled indoles were not present

*Determination of endogenous indoles* For the determination of tryptophan in the Dowex eluate a modification of the fluorimetric method devised by Hess and Udenfriend (19??) was used Thus internal tryptophan standards were used and tissue blanks prepared by adding $H_2O$ before formaldehyde and by omitting the incubation step Endogenous 5 HT present in the Amberlite eluate was determined fluorimetrically according to Bogdanski *et al* (19??) with internal standard The content of endogenous 5 HIAA in the Sephadex eluate was

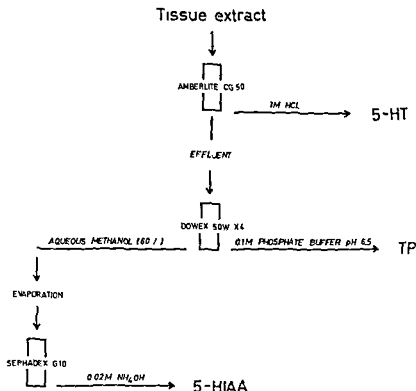



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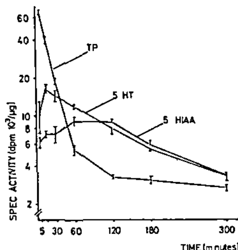



Fig 3 Specific activities of tryptophan (TP) 5 HT and 5 HIAA in rat brain at various time intervals after an i v injection of $^{3}H$ tryptophan (89 $\mu$Ci) Each point represents the mean value ±S E of 4—5 animals

TABLE II Effect of probenecid on contents of labelled 5 HT and 5 HIAA formed from H tryptophan in brain Probenecid was administered i p 10 min before (200 mg/kg) and 60 min after (100 mg/kg) an i v injection of $^{3}H$ tryptophan (95 $\mu$Ci) Groups of animals were killed 60 and 120 min after H tryptophan administration Figures represent mean value±S E from 8—9 animals

| Treatment | Time after H tryptophan injection min | H Tryptophan dpm×10 /g | H 5 HT dpm×10 /g | H 5 HIAA dpm×10 /g |
|---|---|---|---|---|
| Saline | 60 | 21±1 | 3 1±0 4 | 2 7±0 4 |
| Probenecid | 60 | 56±2[1] | 5 8±0 6 | 7 2±1 2 |
| Saline | 120 | 14±1 | 2 1±0 1 | 2 8±0 3 |
| Probenecid | 120 | 35±2 | 3 3±0 4 | 8 6±1 5 |

D ffers from sal ne group (p < 0 001)
D ffers from saline group (p < 0 01)

TABLE III Effect of probenecid on concentrations of endogenous tryptophan 5 HT and 5 HIAA in brain Sal ne or probenecid was dmini tered p 130 m n (200 mg/kg) and 60 min (100 mg/kg) befo e sac ifice of the animals Figures represent mean v lue±S E from 8—9 animals

| Treatment | Tryptophan µg/g | 5 HT µg/g | 5 HIAA µg/g |
|---|---|---|---|
| Sal ne | 2 8±0 1 | 0 27±0 01 | 0 22±0 0? |
| Probenecid | 4 1±0 2 | 0 28±0 02 | 0 41±0 06 |

Differs from saline g oup (p < 0 001)
D ffers from sal ne group (p < 0 01)

## Discussion

Several methods for estimation of 5 HT synthesis and turnover in rat brain are based on the use of pharmacological tools (Tozer Neff and Brodie 1966, Neff, Tozer and Brodie 1967) Such methods may be criticized, since the drugs used have multiple actions on 5 HT metabolism For example monoamine oxidase inhibitors do not only block catabolism of 5 HT but also reduce 5 HT synthesis (Macon Sokoloff and Glowinski 1971) and increase brain tryptophan (Taghamonte *et al* 1971) Probenecid, which blocks transport of 5 HIAA from brain (Neff Tozer and Brodie 1967 Werdinius 1967) also affects levels of tryptophan in brain and serum (Taghamonte *et al* 1971 Korf, van Praag and Sebens 1972)

The use of labelled tryptophan in tracer amounts for the study of 5 HT metabolism has little influence on physiological conditions This technique has been applied for calculation of rates of 5 HT synthesis and turnover by determination of precursor product relationships between specific activities of tryptophan and 5 HT (Lin *et al* 1969 Neff *et al* 1971) The calculations were based on steady state kinetics of endogenous 5 HT Since several drugs and experimental conditions affect levels of tryptophan and 5 HT these quantitative methods are not valid for many pharmacological experiments When concentrations of the endogenous indoles are changing and rates of formation and degradation of 5 HT are dissociated it is necessary to study the metabolic steps involved in both synthesis and catabolism of 5 HT in order to obtain proper information regarding the metabolic change

In the present work such a procedure is offered by the possibility of determining the *in vivo* formation of $^3$H 5 HT as well as its main metabolic product $^3$H 5 HIAA in brain after i v administration of $^3$H tryptophan According to precursor product kinetics in an open single compartment the specific activity of the product should equal that of the precursor at the time when the product reaches its maximal specific activity (Zilversmit Entenman and Fischler 1943) The specific activity time curves of 5 HT and 5 HIAA in Fig 3 seem to fulfill this criterion The same would seem to be the case for the precursor product relationship of tryptophan and 5 HT specific activities which is similar to that previously presented by Neff *et al* (1971) These findings indicate that during physiological conditions the sum of 5 HT stores in brain acts approximately like an open single compartment However the data do not exclude the possibility that 5 HT is contained in more than one compartment within central neurons as suggested by Moir and Eccleston (1968) Grahame Smith (1971 Macon Sokoloff and Glowinski (1971) Bedard Carlsson and Lindqvist (1972 and Shields and Eccleston (1972 1973)

During acute treatment with probenecid the endogenous concentrations of tryptophan and 5 HIAA in brain change (Table III) Thus quantitative measurement of 5 HT synthesis and turnover by use of steady state kinetics cannot be made In probenecid treated animals labelled 5 HIAA accumulated in brain and its concentration was about 3 times that of controls 120 min after $^3$H tryptophan injection (Table II) In agreement with Taghamonte *et al* (1971) and Korf van Praag and Sebens (1972) the drug also increased brain tryptophan Since tryptophan

hydroxylase is not saturated at concentrations of tryptophan normally present in brain (Fernstrom and Wurtman 1971 Friedman Kappelman and Kaufman 1972), the increase in brain tryptophan probably enhances 5 HT synthesis According to Korf van Praag and Sebens (1972) probenecid does not alter 5 HT synthesis since the accumulation of 5 HT after monoamine oxidase inhibition was not altered by probenecid However since monoamine oxidase inhibitors have been shown to inhibit formation of 5 HT (Macon Sokoloff and Glowinski 1971 Hamon *et al* 1972) and 5 hydroxytryptophan (Carlsson and Lindqvist 1972) the effect of probenecid on 5 HT synthesis determined by this method might not appear This is further substantiated by the present finding that probenecid treatment significantly increased the content of $^3$H 5 HT formed from $^3$H tryptophan (Table II)

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# Intracortical Distribution of Renal Blood Flow during Saline Infusion in Dogs

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## Abstract

Løyning E W *Intracortical distribution of renal blood flow during saline infusion in dogs* Acta physiol scand 1974 *90* 409—416

The increase in sodium excretion induced by isotonic saline infusion has been attributed to a redistribution of blood flow within the cortex to salt loosing superficial nephrons To test this hypothesis *mean blood flow to outer (OCF) and inner (ICF) cortex was measured in dogs* before and during saline expansion using $H_2$-clearance rates as indicator of local blood flow Mean total blood flow (RBF) was measured with an electromagnetic flowmeter During infusion of 0 9 % NaCl 30 ml/min for 30—80 min urine flow increased from 1 1 to 7 5 ml/min glomerular filtration rate from 39 8 to 49 1 ml/min RBF rose from 214 to 276 ml/min OCF from 3 43 to 3 87 ml/min and ICF from 3 50 to 3 91 ml/min OCF/ICF did not change significantly during saline expansion Autoregulation of local blood flow was maintained after saline loading with no change in OCF/ICF within the pressure range of autoregulation Since massive saline infusion is followed by a parallel increase in blood flow to outer and inner cortex it is concluded that intracortical redistribution of blood flow is not the mechanism of natriuresis during saline loading


The increase in sodium excretion following infusion of isotonic saline has been attributed to redistribution of renal cortical blod flow from salt retaining nephrons in the deep layer of cortex to salt loosing nephrons in the superficial cortical layer (Barger 1966 Barton *et al* 1968) In support of this hypothesis Barton *et al* (1968) observed *a disproportionate increase in superficial* cortical blood flow *during saline* infusion whereas Munck De Bono and Mills (1970) found no significant difference in the ratio of superficial cortical blood flow to overall cortical blood flow

To reinvestigate this controversial problem blood flow was measured in the renal cortex of dogs 1—3 mm and 6—8 mm from the renal capsule before and during saline infusion using the hydrogen clearance technique previously described by Aukland Bower and Berliner (1964) and Aukland (1968) and modified to measure renal cortical blood flow by Løvning (1971)

Redistribution of blood flow between the superficial and deep parts of cortex implies that vascular resistance should decrease to different degrees in the two

TABLE I Cortical blood flow during saline infusion in one dog

| Time | AP | RBF | | Outer cortical blood flow E₁ | E₄ | E₅ | OCF | |
|---|---|---|---|---|---|---|---|---|
| min | mm Hg | ml/min | | ml/min g | ml/min g | ml/min g | ml/min g | % |
| −2 | 140 | 170 | 100 | 3.08 | 3.42 | 3.55 | 3.35 | 100 |
| 0 | Start infusion of NaCl 0.9 % 30 ml/min | | | | | | | |
| 14 | 135 | 180 | 106 | 3.12 | 3.64 | 3.50 | 3.42 | 102 |
| 25 | 135 | 180 | 106 | 3.17 | 3.80 | 3.30 | 3.4[illegible] | 102 |
| 33 | 135 | 180 | 106 | 3.20 | 4.05 | 3.35 | 3.53 | 106 |
| 50 | 140 | 190 | 112 | 3.36 | 4.80 | 3.75 | 3.97 | 110 |

regions If this were the case the autoregulating capacity of the two regions might be affected differently Local blood flow in superficial and deep cortex was therefore measured at various perfusion pressures before and after saline expansion

## Methods

The distribution of blood flow and the pressure flow relationship in the renal cortex were investigated before and during saline expansion in mongrel dogs weighing 18—27 kg The animals had free access to water but food was withheld for 19 h before operation Pentobarbital was given i v 25 mg/kg b wt in one dose initially and thereafter in repeated doses of 30—60 mg during the experiment, to maintain a light anesthesia. Tracheal intubation secured free airways Catheters were inserted into the femoral artery and vein for pressure recording and saline infusion respectively Retroperitoneal access to the right or left kidney was made through a subcostal flank incision and the renal artery was freed of surrounding fat with care to avoid damage to the renal nerves A flowmeter probe 3—4 mm in diameter depending on vessel size was applied to the renal artery near its origin and mean total renal blood flow (RBF) was measured with a square wave electromagnetic flowmeter (Nycotron Drammen) and recorded on a Sanborn recorder Calibration of the probe had previously been performed on femoral and carotid arteries Zero flow was checked during the experiments by occluding the renal artery for 5—10 s. For urine sampling a polyvinyl catheter was placed in the renal pelvis through an incision in the upper part of the ureter

A polyvinyl tube (0.4 mm external diameter) was inserted in upstream direction in a side branch of the renal artery ad modum Herd and Barger (1964) for injection of hydrogen saturated saline and in [illegible] animals for recording mean renal arterial pressure (RAP) Mean arterial pressures were measured with Statham transducers and recorded on a Sanborn recorder RAP could be reduced in steps with an adjustable clamp on the renal artery between the flow probe and the tip of the polyvinyl tube ([illegible] dogs) or on the aorta above the origin of the renal arteries ([illegible] dogs) In the latter RAP was estimated by subtracting 10 mm Hg from mean aortic pressure (AP) (Løyning [illegible])

Hydrogen [illegible] was used as indicator for local blood flow (Aukland, Bower and Berliner 1964, Aukland 1968, Løyning 1971) Hydrogen concentration in the renal cortex was measured with platinum electrodes connected to a 6-channel polarograph and recorded on a Rikadenki [illegible] channel recorder (Rikadenki Co Tokyo model B64) using a polarizing potential [illegible] versus a KCl saturated calomel reference electrode of 0.2 V Clearance [illegible] (Aukland 1968) To calculate [illegible] the hydrogen saturation curves as previously described (Aukland 1968) To obtain a [illegible] concentration of H before desaturation 10—[illegible] ml H-saturated saline warmed to [illegible] temperature was injected into the renal artery The [illegible] platinum electrodes were inserted 1—3 mm and 6—8 mm into the cortex three electrodes at each level Their tapered tips [illegible] 10 mm in length and 0.[illegible] mm in largest diameter The external part of the [illegible] was stitched to the capsule The kidney was replaced in its normal position and the wound closed

| Inner cortical blood flow | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| E | E | E | ICF | | OCF/ | GFR | V | V |
| ml/ min g | ml/ min g | ml/ min g | ml/ min g | % | ICF | ml/ min | ml/ min | % of GFR |
| 4 25 | 4 25 | 2 83 | 3 78 | 100 | 0 89 | 35 | 0 8 | 2 3 |
| 4 15 | 4 82 | 3 00 | 3 99 | 106 | 0 86 | 42 | 3 2 | 7 6 |
| 4 00 | 4 70 | 2 93 | 3 88 | 103 | 0 88 | — | — | — |
| 4 00 | 4 25 | 3 16 | 3 80 | 100 | 0 93 | 46 | 6 4 | 13 9 |
| 4 30 | 4 82 | 3 46 | 4 19 | 111 | 0 95 | 47 | 5 7 | 12 1 |

AP mean aortic pressure RBF mean renal blood flow $E_1$ $E_2$ E single electrode measurements in outer cortex OCF mean of E $E_2$ E E $E_4$ E single electrode measurements in inner cortex ICF mean of E E E GFR glomerular filtration rate V urine flow

A priming (10 ml) and sustaining (2 ml/min) solution of creatinine or $^{51}$Cr EDTA in 0 9 % saline were administered i v to give plasma concentrations of about 15 mg % for creatinine and suitable counting statistics for $^{51}$Cr EDTA Creatinine was measured by the method of Bonsnes and Taussky (1945) and $^{51}$Cr EDTA was counted on equal volumes of diluted urine and plasma in a well type scintillation counter (Stacy and Thorburn 1966 Eide 1970)

Measurements were started 60—90 min after completion of the surgical procedure and the administration of the sustaining solution H -clearance was measured and blood and urine samples were collected prior to the infusion of 0 9 % NaCl i v at a rate of 30 ml/min for 30—80 min During the saline infusion H -clearance was measured and blood and urine samples were collected at intervals of 10—20 min In order to compare local blood flow in outer and inner cortex (OCF and ICF respectively) in different experiments the data from all electrodes in one cortical region were averaged and calculated *in per cent of the last control measure*ment before saline infusion was started

The flow/pressure relationship was studied in 4 dogs before the infusion of saline RAP was reduced in steps and H clearance was measured at each step One or two measurements were made within the pressure range of autoregulation and usually one measurement at a perfusion pressure well below this range This procedure was repeated after saline infusion when an increase in RBF and urine flow was no longer observed during a 10 min period

At the end of the experiment the renal pedicle was clamped and the kidney removed The position of the electrodes was checked and the results were discarded if the electrodes were macroscopically in contact with calyces

Statistical evaluation of the results with significance level of 5 % was made using Students t test for paired comparison between mean blood flow in outer and inner cortex (OCF and ICF respectively) and for covariance analysis of the flow ratio OCF/ICF and urine flow in per cent of glomerular filtration rate and of OCF/ICF and renal perfusion pressure (Snedecor 1967)

## Results

*Saline infusion* Data from a typical experiment is shown in Table I During 30—80 min of saline infusion RBF increased on average from 214 to 276 ml/min GFR from 40 to 49 ml/min and V from 1 1 to 7 5 ml/min In control period outer cortical blood flow (OCF) was 3 43 ml/min and inner cortical blood flow (ICF) 3 5 ml/min increasing to 3 87 and 3 91 ml/min a percentage increase of 13 % and 12 % respectively No significant difference between blood flow in superficial and deep cortex was found neither in control nor after saline infusion

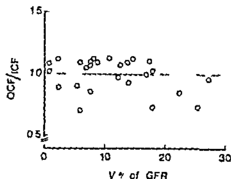



*Fig 1* Relation between urine flow in per cent of glomerular filtration rate (V % of GFR) and distribution of cortical blood flow (fraction of outer on inner cortical blood flow = OCF/ICF)

Fig 1 shows the relationship between urine flow in per cent of glomerular filtration rate (V in % of GFR) and the ratio OCF/ICF An analysis of covariance showed no significant differences among the slopes obtained in the 6 dogs

*Autoregulation* Autoregulation of local blood was similar at all electrode sites in the superficial and deep layer of cortex both before and after saline infusion The flow/pressure relationship before and after saline expansion is presented in Fig 2 Each plot represents the average of 1—3 electrode measurements at that particular perfusion pressure Parallel changes in local and total blood flow were observed and

Fig 2 Pressure/flow relationship before (○) and after (●) saline expansion (RBF renal blood flow OCF outer cortical blood flow ICF inner cortical blood flow AP mean aortic pressure RAP mean renal arterial pressure)

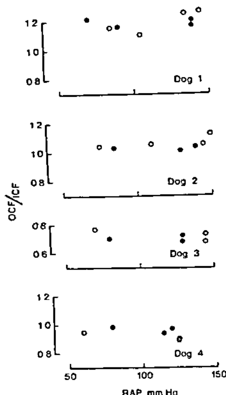



Fig 3 Relation between cortical blood flow distribution (OCF/ICF) and mean renal arterial pressure (RAP) before (O) and after (●) saline infusion within the pressure range of autoregulation

the saline infusion did not modify this relationship Autoregulation of RBF was slightly better in control experiments than after saline infusion Before saline infusion RBF was reduced by less than 1 % when RAP was lowered to 72 5 mm Hg OCF and ICF both fell to 91 % of control After a period of 44 to 78 minutes of salt loading RBF was reduced by 5 % when RAP was lowered to 79 mm Hg OCF and ICF decreased to 86 % of control

In Fig 3 the flow ratio OCF/ICF is related to RAP in control condition (open circles) and after salt loading (closed circles) at perfusion pressures with less than 12 % reduction in RBF (lowest limit of RAP was on average 70 mm Hg) A common standard deviation was estimated by pooling the data from all 8 lines and the slopes before and after saline expansion were not significantly different in 3 dogs whereas in dog 1 the difference was significant

## Discussion

The increase in renal blood flow and urine flow following saline infusion was accompanied by proportional increases in the rates of $H_2$-clearance from superficial and deep parts of the renal cortex No difference in autoregulation of local blood flow in the two regions of the cortex was observed after saline loading

Extensive studies of the $H_2$ clearance technique and a thorough discussion of its validity for estimating local blood flow have been made previously by Aukland Bower and Berliner (1964) Neely *et al* (1965) and Aukland (1968) and particularly concerning cortical blood flow by Loyning (1971) and Aukland *et al* (1973) who found monoexponential curves down to at least 90 % desaturation Monoexponential curves of similar quality were also observed in the present experiments both before and after saline expansion Furthermore good agreement between results obtained by the $H_2$ clearance method and those obtained by measuring local clearance of $^{85}$Kr and mean transit time of $^{32}$P labelled erythrocytes in the dog kidney has been reported by Aukland and Wolgast (1968)

Considerations of volume changes in the kidney seem warranted however, since blood flow per volume tissue as indicated by the rate constant of $H_2$ desaturation would increase less than total blood flow when tissue volume increases Saline infusion of 20—50 ml/min may cause enlargement of the distance between two piezoelectric crystals in the cortex from 4 % (Kiil Johannesen and Aukland 1971) to 8 % (Omvik Ræder and Kiil 1971) corresponding to a volume increase of about 12 to 26 % At the largest volume increase RBF rose to 160 % of control (Omvik *et al* 1971) Maximal dilatation of autoregulatory elements is expected when saline expansion has caused more than doubling of the renal blood flow (Kiil Kjekshus and Loyning 1969) In the present experiments RBF increased on average to 129 % of control after saline expansion with autoregulation still present indicating moderate expansion and kidney swelling Assuming therefore a kidney swelling to 115 % of control the rate constant of $H_2$-clearance from the cortex would increase to only 112 % of control in good agreement with the present results (113 % and 112 % in outer and inner cortex respectively)

The difference between local blood flow in outer and inner layer of cortex was not significant at the 5 % level neither in control (as also demonstrated previously by Loyning 1971 and Aukland *et al* 1973) nor after saline expansion indicating a uniform distribution of blood flow within the cortex provided the swelling of the cortex was uniform

An increase in outer cortical blood flow escaping detection with the hydrogen washout technique because of a disproportionate increase of outer cortex volume would imply a greater reduction in vascular resistance and consequently in autoregulating capacity of this region In the present experiments the regression coefficient for OCF ICF related to renal perfusion pressure before and after saline infusion Fig 3 were not significantly different in three dogs The autoregulation of local blood flow therefore was similar in outer and inner cortex suggesting that saline expansion causes a uniform increase in cortical volume and therefore in local blood flow If not by mere coincidence the significant difference in regression coefficient shown by dog 1 would indicate a change in the capacity of autoregulation opposing the redistribution hypothesis since the regression coefficient became more close to zero after saline infusion

Antiglomerular basement membrane antibody has recently been introduced by Wallin Rector Jr an Seldin (1971) seemingly as an almost ideal indicator of intra renal plasma flow Their observations indicating that plasma flow is uniformly distri buted within the renal cortex in hydropenic dogs and in dogs in a state of moderate saline expansion are in agreement with the present results and others previously published (Munck De Bono and Mills 1970 Loyning 1971 Aukland *et al* 1973) but the observed redistribution of plasma flow to deeper glomeruli during massive and continuous infusion of isotonic saline was not confirmed by the present ex periments The results are not quite comparable however since the antibody technique measures glomerular plasma flow and the hydrogen clearance technique presumably measures blood flow mainly in the postglomerular capillary bed Under estimation of plasma flow with the antibody technique must be taken into account during saline expansion since the number of glomeruli per volume unit being highest in the superficial cortex (Horster Kemler and Valtin 1971) decreases when kidney volume increases

Blood flow in outer and inner cortex in relation to perfusion pressure after saline infusion has not previously been studied Total renal blood flow is increased during saline infusion and the disproportionate increase in blood flow to outer cortex sug gested by the redistribution hypothesis implies greater arteriolar dilatation in outer than in inner cortex with greater sensitivity to variations in perfusion pressure The present experiments therefore showing autoregulation of blood flow of similar ca pacity in the two regions are strong evidence against redistribution of blood flow to the superficial renal cortex during saline loading

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# Influence of Aldosterone on Active Sodium Transport by the Toad Bladder a Kinetic Approach

B

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## Abstract

CRABBE J *Influence of aldosterone on active sodium transport by the toad bladder a kinetic approach* Acta physiol scand 1974 *90* 417—426

Sodium transport by the urinary bladder of the toad *Bufo marinus* was analysed by measuring the rate of transfer of radiosodium from the tissue towards the serosal compartment at steady state with respect to labelling from the mucosal surface Two sodium pools were identified of which only the fast-disposal pool (with a half time of 1 7 min) reflected the sodium transporting activity of the preparation. When transepithelial sod um transport was stimulated by aldosterone the half time of the fast-disposal sodium pool was not modified This is taken as additional evidence for the assumption that aldosterone does not influence the handling of sodium by the "pump

The mechanism whereby aldosterone stimulates active sodium transport across epithelia such as amphibian bladder and skin (Crabbe 1963) is still controverted Some investigators have concluded that the hormone acts on the energy requiring step which supposedly operates at the basal pole of these epithelial cells or at their borders facing interspaces or at both Others believe that the stimulation of sodium transport by aldosterone results from changes at the apical pole which sodium penetrates upon interaction with pecific carrier molecules (Sharp and Leaf 1966 Edelman and Fanestil 1970 Crabbe 1972)

To shed additional light on the issue experiments have been conducted with toad bladder relating by means of tissue analysis the size of the pool of sodium involved in active transport to the rate of active transport Assuming the sodium pool to be homogeneous and the ion to be disposed of by the tissue according to first order kinetics a half time of 2—3 min could be calculated for the pool This half time was not modified by aldosterone (Crabbe and De Weer 1969)

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The problem of the localization of this tissue sodium pool however arose for all sodium originating from the outside and having crossed the apical border of the epithelial cells was measured—i e sodium already expelled by the pump located in the epithelial cells together with sodium proximal to that pump Therefore a kinetic approach was applied to study sodium transport by toad bladder It was found that the half time of the pool of tissue sodium involved in transepithelial sodium transport was 1 7 min and that it was not influenced by aldosterone

Thus both the tissue analysis and the kinetic approach yield data compatible with the assumption that aldosterone does not act directly on the pump

However even with the kinetic procedure adopted it could not be shown unequivocally that all sodium forming the tissue pool dealt with is proximal to the sodium pump

## Methods

Toads *Bufo marinus* were used after a captivity of up to two months without feeding They were kept in shallow water for a few days before sacrifice and several animals received the preceding evening a subcutaneous injection of 10—20 μg aldosterone dissolved in 0 2—0 5 ml saline After pithing both bladder halves were dissected free and cut open longitudinally by an incision along the dorsal surface of the organ so as to avoid stretching of the tissue when the latter was set up for incubation One membrane was used immediately while the second bladder half was stored in unstirred Ringer's fluid at 4 C for 24—48 h Conservation was adequate as judged from short circuit current and transmembrane potential measurements means were 34 μA/cm and 43 mV respectively after 1—2 h of incubation at room temperature following storage against 30 μA/cm and 46 mV for the matched bladder halves studied immediately N = 10 pairs)

The experimental procedure was adapted from Andersen and Zerahn (1963) The bladder was placed in the incubation chamber (incubation area 7 cm ) with the mucosal surface facing upward while the serosal surface was resting on a piece of stretched nylon stocking When potential and current were stable the solution containing a radioisotope of sodium was introduced in the upper compartment corresponding to the apical border of the epithelium and the membrane was maintained short circuited After at least 15 minutes the labelled solution was removed and the mucosal surface of the bladder was rinsed for 30 s or 60 s with distilled water flowing across the upper part of the incubation chamber at a rate approximating 200 ml per min Residual fluid was drained as thoroughly as possible from the mucosal surface (without touching the preparation!) and counts were recorded with a G M tube facing the mucosal surface of the membrane at 1 min intervals for 30 min Generally 60 to 90 s elapsed between interruption of short-circuiting and the start of the counting by means of which the rate of disappearance of radiosodium from the incubation chamber could be followed As the solution in the lower compartment of the chamber is renewed in a few seconds the counting provides data mainly related to disposal of sodium by the tissue towards the inside

Plotted on semi logarithmic coordinates the experimental data yield 2 straight lines from which the half time of 2 sodium pools can be determined The size of both sodium pools is arrived at from extrapolation to the moment the solution was removed from the outside compartment and from the specific activity of sodium in the loading solution With pool size (in μEq Na and its half time (in min) sodium flux (in μEq Na per hour) can be computed

$$\text{Flux} = \frac{\text{Pool size} \times \ln 2 \times 60}{\text{Pool half time}}$$

Flux values associated with the fast-disposal pool (pool A) only are comparable to those derived from short-circuit current measured immediately before removal of the loading solution

The tissue sodium pool was measured in a few instances by another kinetic method whereby one determines the time required for sodium influx to reach a steady state with respect to the net sodium transporting activity of the preparation after introduction of radiosodium on the mucosal side (Hoshiko and Ussing 1960 Andersen and Zerahn 1963) The time constant of this process is the reciprocal of the rate coefficient k and is thus related to the half time

TABLE I Serial determinations of sodium pool size and half time in isolated toad bladder

| Membrane | Time* | Potential Difference (mV) | Short circuit current ($\mu$Eq $Na^+$/h) | Pool A Half time (min) | Pool A Size ($\mu$Eq $Na^+$) | Na influx** ($\mu$Eq $Na^+$/h) | Tissue Weight (mg) |
|---|---|---|---|---|---|---|---|
| 1 | 11 40 | 27 | 3 99 | 2 0 | 0 142 | 2 94 | |
| | 14 00 | 23 | 2 39 | 1 7 | 0 099 | 2 40 | |
| | 17 00 | 16 | 1 57 | 1 6 | 0 091 | 2 34 | 85 4 |
| 2 | 12 00 | 47 | 7 35 | 2 0 | 0 221 | 4 60 | |
| | 13 35 | 55 | 8 15 | 1 9 | 0 314 | 6 85 | |
| | 15 50 | 51 | 5 50 | 2 3 | 0 201 | 3 64 | 98 1 |
| 3 | 13 25 | 33 | 5 70 | 1 5 | 0 167 | 4 60 | |
| | 14 00 | 36 | 5 60 | 1 5 | 0 203 | 5 60 | |
| | 14 40 | 32 | 4 65 | 1 8 | 0 168 | 3 88 | 90 7 |
| 4 | 13 45 | 28 | 4 58 | 2 2 | 0 164 | 3 09 | |
| | 15 00 | 22 | 3 10 | 2 0 | 0 192 | 3 97 | |
| | 19 15 | 24 | 4 30 | 2 4 | 0 191 | 3 30 | 168 8 |

* The time given is that of the last short-circuit current reading

** Computed from half time and size of pool A (see Methods)

N B [$Na^+$] in Ringer's on the outside was 11 5 mEq/l The first 2 preparations were studied with Na the latter 2 with Na

of pool A $\tau = \frac{t\,1/2}{\ln 2}$ $\tau$ is obtained graphically from the intersection of the linear part of the "build up" curve with the x (*i e* time) axis when extrapolated downward *i e* to y = 0 (y standing for radioactivity scale)

Frog Ringer's (NaCl 115 mM $KHCO_3$ 2 5 mM $CaCl_2$ 1 mM) was used on the serosal side on the mucosal side it was often diluted 10 times with an equivalent solution in which sodium was replaced by magnesium ($MgCl_2$ 57 5 mM $KHCO_3$ 2 5 mM $CaCl_2$ 1 mM)

A number of preparations were examined after cooling (3 expts ) or after treatment with metabolic inhibitors 2 4 dinitrophenol $3\ 5\times10^{-?}$ M and $10^{-4}$ M (5 expts ) sodium cyanide 0 5 and 2 0 mM (4 expts ) ouabain $5\times10^{-5}$ M (10 expts ) These drugs were added on the inside care being taken to maintain solution pH at 7 8

The incubated tissue was weighed after completion of the studies

## Results

### I *Evaluation of procedure applied to the toad bladder*

a) *Stability of the preparation* The kinetic method described for the study of sodium transport by frog skin (Andersen and Zerahn 1963) proved harmless for toad bladder as determinations can be carried out several times over several hours (Table I)

b) *Dilution of sodium in the solution used on the outside* Attemps to evaluate sodium transport by the isolated toad bladder met with difficulty when the radioisotope was added to straight Ringer's solution because of the frequent occurrence of high background radioactivity This appeared as the component with a long half time (pool B) When sodium in Ringer's was diluted 10 times sodium transport activity was only moderately decreased (Table II) and the fast component of the

TABLE II Active sodium transport by the isolated toad bladder effect of dilution of sodium in loading solution (Means ± S E )

| | Ringer s (115 mEq $Na^+$/L) | Dilute Ringer s (11 5 mEq $Na^+$/L) |
|---|---|---|
| Number of membranes studied | 6 | 18 |
| Number of determinations | 10 | 36 |
| Final current ($\mu$Eq $Na^+$/h) | 9 93 ± 1 34 | 4 73 ± 0 59 |
| Membrane potential (mV) | 55 ± 12 | 26 2 ± 3 1 |
| *Pool A* | | |
| Half time (min) | 1 66 ± 0 13 | 1 73 ± 0 07 |
| Size ($\mu$Eq $Na^+$) | 0 338 ± 0 058 | 0 140 ± 0 018 |
| $Na^+$ influx ($\mu$Eq $Na^+$/h) | 9 34 ± 1 87 | 3 49 ± 0 51 |
| Ratio influx/current* | 0 806 {0 653, 0 995} | 0 728 {0 679, 0 781} |
| *Pool B* | | |
| Half time (min) | 32 ± 4 | 19 6 ± 1 3 |
| Size ($\mu$Eq $Na^+$) | 0 278 ± 0 075 | 0 041 ± 0 003 |
| *Tissue weight (mg)* | *62 4 ± 6 5* | *74 8 ± 6 5* |

* Computed from log of individual ratios

N B 1 Except for 1 membrane of the series exposed to Ringer s all were from toads treated with aldosterone

2 Membranes were always rinsed with $H_2O$ for 60 s after exposure to Ringer s rinsing time was 60 s in 1/3 of the cases after exposure to modified solution when it became apparent that little could be gained by flushing $H_2O$ for more than 30 s rinsing lasted 30 s only in the other 2/3 of this series

curve could be defined more accurately without modification of its half time On the other hand the decrease of size of the pool computed from the background curve (pool B) was almost proportional with the degree of dilution

(c) *Quenching* Net sodium flux derived from short circuit current usually exceeded sodium influx computed from size of pool A and its half time when $^{24}$Na was used for labelling This results from quenching of $^{22}$Na emission when measured with a G M tube rather than from an intrinsic defect of the method since there was agreement when $^{24}$Na was used instead with this more energetic $\beta$ emitter the sodium influx amounted to 0 98 ± 0 06 (S E ) of the final short circuit current (12 expts)

Equivalence between electrical and radioisotopic data for transepithelial sodium transport should be expected even when sodium is diluted ten fold on the mucosal surface of the toad bladder (Crabbé and De Weer 1969)

*II Effect of aldosterone on sodium transport by the toad bladder*

Under the influence of aldosterone potential and current were increased about 50% above the baseline (Table III) The half time of pool A was unchanged and the size of pool A as well as computed sodium influx increased to the same extent as the current Also pool B increased after aldosterone treatment

TABLE III Effect of aldosterone on parameters of active sodium transport by the isolated toad bladder (means ± S E )

| | Untreated | + Aldosterone |
|---|---|---|
| Number of membranes studied | 6 | 18 |
| Number of determinations | 10 | 36 |
| Final current (μEq Na⁺/h) | 3 14±0 52 | 4 73±0 59 |
| Membrane potential (mV) | 15 3±2 6 | 26 2±3 1 |
| *Pool A* | | |
| Half time (min) | 1 76±0 11 | 1 73±0 07 |
| Size (μEq Na⁺) | 0 099±0 015 | 0 140±0 018 |
| Na⁺ influx (μEq Na⁺/h) | 2 43±0 36 | 3 49±0 51 |
| Ratio influx/current | 0 783 {0 659, 0 931} | 0 728 {0 679, 0 781} |
| *Pool B* | | |
| Half time (min) | 23 0±2 5 | 19 6±1 3 |
| Size (μEq Na⁺) | 0 025±0 007 | 0 041±0 008 |
| Tissue weight (mg) | 77 1±6 5 | 74 8±6 5 |

* Computed from log of individual ratios
N B All experiments were carr ed out with * Na added to Ringer s fluid containing 11 5 mEq Na⁺/l

TABLE IV Effect of metabolic inhibitors on parameters of active sodium transport by the isolated toad bladder (Mean values)

| | Cooling* N = 3 | | D n trophenol N = 5 | | Cyanide ** N = 4 | | Ouabain* ** N = 6 | |
|---|---|---|---|---|---|---|---|---|
| | Before | During | Before | Dur ng | Before | During | Before | Dur ng |
| Final current (μEq Na⁺/h) | 2 84 | 1 27 | 3 50 | 0 99 | 7 50 | 2 73 | 5 11 | 1 69 |
| Membrane potent al (mV) | 25 | 17 | 19 | 6 | 37 | 21 | 29 | 16 |
| *Po l A* | | | | | | | | |
| Half t me (m n) | 1 6 | 2 5 | 2 1 | 2 0 | 1 5 | 3 1 | 1 8 | 3 1 |
| S ze (μEq Na⁺) | 0 101 | 0 104 | 0 168 | 0 081 | 0 221 | 0 122 | 0 164 | 0 078 |
| Na⁺ influx (μEq Na⁺/h) | 2 52 | 1 73 | 3 74 | 1 7? | 6 73 | 1 73 | 3 78 | 1 09 |
| *Po l B* | | | | | | | | |
| Half time (min) | 21 | 16 | 29 | 25 | 25 | 26 | 17 | 12 |
| Si e (μEq Na⁺) | 0 023 | 0 040 | 0 024 | 0 024 | 0 029 | 0 127 | 0 0?7 | 0 074 |

from 25 C to 12 C

* 3 5 to 10×10 M on the n de

0 5 to 2×10 M on the n de

* 5×10 M on the ns de

N B All experiments we arri d out with Na added to R nger s fluid containing 11 5 mEq Na⁺/l

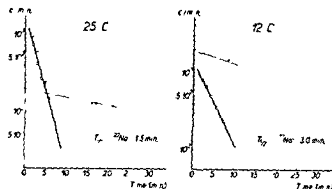



Fig 1 Effect of cooling on the sodium transporting activity of the toad bladder A drop of temperature from 25 C to 12 C resulted in a two-fold increase in the half time of the initial component (0 0 pool A) of the experimental curve describing the disappearance of radiosodium from the preparation towards its serosal surface as a function of time

### III *Effects of inhibitors on active sodium transport by the toad bladder*

Neither dilution of sodium in the loading solution nor stimulation of sodium transport by aldosterone resulted in changes in the half time of tissue sodium pool A The question therefore arose of the location of pool A which could be made up of sodium already beyond the active transport mechanism under focus (Zerahn 1969) If this were the case the half time of pool A would be expected to remain unmodified under most circumstances But if a significant fraction of tissue sodium pool A is proximal to the pump inhibitors of the latter are expected to slow down sodium disposal by the tissue

To examine this alternative sodium transport was inhibited in several ways Control data were secured and the reversibility of the action of the inhibitor on current and potential was assessed

a) Tissue sodium pool size and half time were measured in 3 instances first at room temperature ($\pm 25°$ C) then at a temperature of 12° C It can be seen (Table IV) that short-circuit current was thereby decreased by more than 50 % while the

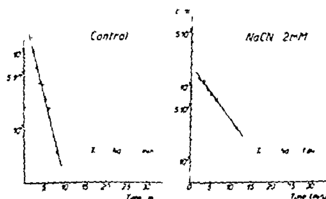



Fig 2 Influence of cyanide on active transepithelial sodium transport by the isolated toad bladder After examination of the preparation in baseline conditions whereby a half time for pool A of 1 4 min was obtained (initial component 0-0) sodium cyanide was added to the solution to which the serosal surface of the tissue was exposed Another analysis of the kinetics of sodium transport was carried out in the presence of the inhibitor with a results a lengthened half time for pool A and a 4 fold increase in the size of pool B

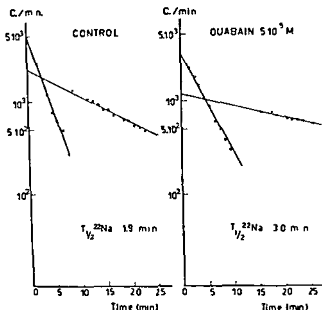



Fig 3 Effect of ouabain on parameters of sodium transport by the isolated toad bladder Exposure of the preparation to this inhibitor of sodium transport resulted in a lengthening of the half time for pool A (0-0) without increase in its size

half time of pool A increased from 1 6 to 2 5 min the size of this tissue sodium pool was uninfluenced One such experiment is described graphically in Fig 1

b) When cyanide inhibited sodium transport the half time of pool A increased (Table IV Fig 2) Yet the size of this pool decreased when compared with controls

c) Analogous studies were performed with ouabain Again half time of tissue sodium pool A increased unlike its size (Table IV Fig 3)

As was already the case with cyanide residual radioactivity (pool B) increased sizably in the inhibited preparation often preventing detection of the steep part of the curve to be analyzed Because of this difficulty in 4 additional expts carried out with ouabain the time constant for tissue sodium pool (see Methods) was measured instead Ouabain brought about a lengthening of $\tau$ from a mean of 3 3 min in base line conditions to 4 9 min when sodium transport had decreased from 5 0 to 1 9 $\mu$Eq/h

d) 2 4 dinitrophenol did not change the half time of tissue sodium pool A despite a large decrease in current and potential the pool size also decreased albeit to a smaller extent (Table IV) The computed sodium influx became large with respect to short-circuit current and the inhibition was irreversible unlike the effect of other inhibitors of sodium transport

e) Pool B calculated from the slow component of the experimental curve was increased 2—4 times by exposure of toad bladder to cold to cyanide and to ouabain without change in half time Dinitrophenol influenced neither the size of pool B nor its half time

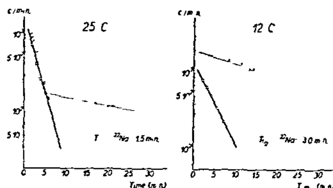



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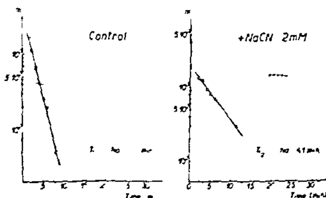



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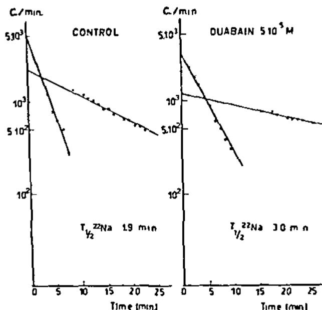



Fig 3 Effect of ouabain on parameters of sodium transport by the isolated toad bladder Exposure of the preparation to this inhibitor of sodium transport resulted in a *lengthening of the half time* for pool A (0-0) without increase in its size

half time of pool A increased from 1 6 to 2 5 min the size of this tissue sodium pool was uninfluenced One such experiment is described graphically in Fig 1

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## Discussion

I *Significance of data provided by the kinetic approach*

Toad bladder, studied with a kinetic method developped for frog skin (Andersen and Zerahn 1963) yields data closely resembling those obtained with frog skin for both epithelia, the curve describing the disappearance of radiosodium from the tissue toward the inside as a function of time can be resolved in 2 exponentials As for frog skin only the steep component (A) of the curve is relevant for analysis of transepithelial sodium transport by toad bladder for

a) there is fair agreement between sodium flux calculated from short circuit current and sodium influx derived from the size of pool A and its half time while no such relationship obtains when pool B is relied upon instead

b) a 10 fold dilution of sodium on the outside has only minor consequences for short circuit current and for sodium influx derived from size and half time of pool A in contrast the size of the slowly disappearing pool (pool B) was decreased by this dilution of external sodium almost in proportion to the degree of dilution

1) *Pool A* Under baseline conditions the half time of this pool averaged 1 7 min with the method applied This value is in agreement with that of 2 min recently reported (Finn and Rockoff 1971) and with that of 2 5 min (Herrera 1965) obtained by another kinetic approach (Schoffeniels 1957) A mean half time slightly in excess of 2 min was calculated on the basis of tissue analysis performed at steady state after exposure to radiosodium of the mucosal surface of the bladder of *Bufo marinus* (Crabbe and De Weer 1969) Frazier and Hammer (1963) on the other hand had concluded also from kinetic studies that the half time for tissue sodium expelled toward the serosal side of toad bladder was 5—7 min a yet longer half time was obtained with the same procedure (Leaf 1966) These large values were reported for preparations examined several minutes after withdrawal of the loading solution

It could be argued that one is dealing exclusively in the case of pool A with sodium already expelled by the epithelial cells—*i e* with a *transported* pool (Zerahn 1969) However no evidence could be provided with the method used for the existence in the case of toad bladder of a yet smaller sodium pool (than pool A) with a yet shorter half time Finn and Rockoff (1971) were likewise unable to demonstrate the existence of such a hypothetical pool

An argument for the presence of sizable amounts of sodium within the epithelial cells rests on the morphological observations of Voute and Ussing (1968) and Voute (1973) indicating that in frog skin increased net sodium transporting activity is accompanied by swelling of the cells involved Furthermore a decrease in the rate of disposal toward the inside occurred for sodium forming pool A when toad bladder was exposed to cold cyanide or ouabain The fact remains however that the size of sodium pool A hardly ever increased concomitantly this might be due to pool A involving in addition to intraepithelial cell sodium—which is proximal to the "pump" —sodium already expelled by the latter in the intercellular paces In frog skin the volume occupied by these interspaces has been shown to bear a direct relationship

to the rate of sodium transport by the tissue (Voute and Ussing 1970) Diamond and Tormey (1966) have observed a swelling of the epithelial cells coinciding with a collapse of the interspaces upon exposure of rabbit gall bladder to ouabain or to cold In addition it is quite conceivable that some interplay operated between the apical border (entry step) and the other borders (extrusion by the pump ) of cells specialized in transepithelial sodium transport preventing large changes in cell volume—and in cell sodium pool size—when one of these step is interfered with (MacRobbie and Ussing 1961) Biber (1971) and Hvid Larsen (1973) have recently provided evidence for a decreased permeability towards sodium of the apical border of the amphibian skin epithelium when the preparation is exposed to ouabain or deprived of oxygen

b) *Pool B* As stated the size of pool B expressed as background radioactivity in the experiments reported is to a large extent a function of the concentration of sodium in the solution corresponding to the mucosal—or apical—surface of toad bladder This suggests that pool B is contributed by residual sodium on the surface of the epithelium Actually when a marker for extracellular space as $^{14}C$ sucrose was added to the solution on the outside $^{14}C$ could be recovered together with some radiosodium after removal of the loading' solution and rinsing of the mucosal surface as described Pool B was markedly and selectively increased in size upon exposure of the toad bladder to agents inhibiting sodium transport such as cold cyanide or ouabain Since more thorough rinsing of the mucosal surface failed to be beneficial under such circumstances this additional sodium is thought to be located within those cells interspersed in the structures supporting the epithelium proper

II *Effect of aldosterone on active transport by the toad bladder*

Aldosterone increased the rate of sodium transport without influencing the rate of disposal of the ion by the epithelial layer of the preparation one would therefore be led to state that this hormone exerts its action at a step involved in transepithelial sodium transport which is distinct from the pump proper The sodium content of isolated epithelial cells of the toad bladder has been recently reported to be increased under the influence of aldosterone (Handler Preston and Orloff 1971 Leaf and MacKnight 1972) Thus conclusions formulated earlier on the basis of tissue analysis (Crabbe and De Weer 1969) are thereby confirmed and an additional argument is provided for the hypothesis that aldosterone acts at the apical pole of the epithelial cells which are targets for the hormone

I wish to thank Professor USSING and his colleagues of the Institute of biological Chemistry for their hospitality and assistance at all stages of this work my gratitude goes more specifically to Dr Karl ZERAHN without whom this study would not have been carried out

It is also a pleasure to acknowledge the valuable comments made by Drs DE WEER HERRERA and MOREL who consented to review this text

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Acta physiol scand 1974 90 427—430

From the Psychopharmacology Research Unit Statshospitalet Risskov Denmark

# Renal Lithium Clearance during Dehydration and Rehydration with Water or 0 9% NaCl in the Rat

By

DONALD F SMITH

Received 29 May 1973

## Abstract

SMITH D F *Renal lithium clearance during dehydration and rehydration with water or 0.9% NaCl in the rat* Acta physiol scand 1974 *90* 427—430

Male albino rats fed a diet containing lithium were dehydrated by 24 h of water deprivation and then rehydrated with demineralized water or 0 9% NaCl Renal lithium clearance decreased during rehydration with water Rehydration with 0 9% NaCl prevented the fall in the rate of urinary lithium elimination The decrease in renal lithium clearance in the rats rehydrated with water was due to an increased fractional reabsorption of lithium in the renal tubules and was associated with a decrease in renal sodium clearance The relationship between the renal elimination of sodium, potassium and lithium during rehydration is discussed

The use of lithium as a drug calls for an understanding of the factors that influence lithium excretion The principal route of lithium excretion is in the urine Adverse side effects of lithium salt therapy can occur when the serum lithium level rises due to a decrease in renal lithium clearance (Schou 1968) It is therefore important to know the factors that decrease renal lithium clearance

A low sodium intake (Schou 1958) lack of adrenal gland hormones (Smith *et al* 1970 Smith and Thomsen 1973) diurnal variations (Smith 1973 a) and strenuous exercise (Smith 1973 b) decrease renal lithium clearance as well as the elimination of other electrolytes Rehydration with water can decrease the renal elimination of sodium and potassium in man (McCance and Young 1944 Aperia *et al* 1969) The relationship between the renal elimination of sodium potassium and lithium (Thomsen *et al* 1969 Smith 1973 a) indicates that rehydration with water may decrease lithium clearance The present study shows that after 24 h of fluid deprivation rehydration with water lead to a decrease in renal lithium clearance while rehydration with 0 9% NaCl prevented the fall in the rate of urinary lithium elimination in rats

Key words lithium clearance dehydration rehydration water sodium chloride kidney function

TABLE I Renal clearance of lithium sodium and creatinine during dehydration and rehydration with demineralized water or 0 9 % NaCl in the rat Values are mean ± S D

| Group | Treatment | Number of rats | Fluid Intake *ml/h* | Urine Volume *ml h* |
|---|---|---|---|---|
| I | Control | 16 | 0 8 | 1 1 ± 0 3 |
| II | Dehydrated | 16 | 0 0 | 0 3 ± 0 2 |
| III | Water Rehydrated | 16 | 2 7 | 1 3 ± 0 3 |
| IV | 0 9 % NaCl Rehydrated | 16 | 4 0 | 3 8 ± 7 3 |
| V | Normally Hydrated + Water Load | 12 | 9 0 | 8 4 ± 0 4 |
| VI | Dehydrated – Water Load | 12 | 9 0 | 5 0 ± 0 8 |

* statistically significant $p < 0.01$

## Methods

28 male albino Wistar rats (320—360 g) were used They were housed in a thermostatically controlled room (24 ) with a 12 h light-dark cycle (lights on 8 AM to 8 PM) and fed at wet mash diet (Thomsen 1970) to which LiCl was added (40 mM lithium/kg dry weight) for 3 days before the clearance tests Food was withh ld from the rats during the cl arance tests

Blood samples (0 2—0 4 ml) were taken under ether anesthesia from the rats tail 30 min before and after the clearance tests The representative serum lithium concentrati n in each rat was computed and used to calculate the clearance values Blood samples for determination of creatinine were taken from the inferior vena cava 30 min after the last clearance test

The rats were induced to empty their bladders at the start and the end of each clearance test by placing them individually on a clear plastic sheet and lifting their tail The urin ex creted at the start of the clearance test was discarded while the urine excreted at the end of the clearance test was added to the collection flask containing the rats urine sampl The metabolism cages were rinsed with enough demineralized water to give a final volume of 25 ml or 50 ml in each rats urine collection flask.

The lithium and sodium content in the serum and urine was determined by flame photometry Creatinine in the serum and urine was determined with the alkaline picrate method after ad sorption on Lloyd s reagent

The first experiment was done to determine the effect of dehydration and voluntary re hydration with water or 0 9 % NaCl on renal lithium clearance 16 of the rats were randomly divided into 4 groups Group I (Control) was not water deprived Group II Dehydrated) was deprived of water for 24 h before the clearance test and also during the test. Group III Water Rehydrated) was deprived of water for 24 h before the clearance test and then was given demineralized water to drink *ad libitum* during the test, and Group IV 0 9 NaCl Reh drated was deprived of water for 24 h before the clearance test and then was given 0 9 % NaCl to drink *ad libitum* during the test. The rats were tested once a week for 4 consecu n weeks New groups were made by random selection of the rats each week Tl learance tests were d ne from 10 AM to PM

The second experiment was done to determine the effect of forced rehydration with a large water l ad n renal l thium clearance In rder to do this a permanent na sa tric tube was in talled in t rats Epstein and Teitelbaum 1962 A cross-over des gn was used t adm er a 9 ml h l ad of demineralized water to 6 of the rats when they were n t water depri ed (Gro V and then after they had been deprived of water for 4 h (Gro p VI Tl treat ments were adm nistered to the other 6 rats in the reverse order Th l ad wa adm n tered a a con tant r b m ans of an infu ion pump The cl arance test were d n e a week for two conse w ks from 11 AM to PM

## Results

The results appear in Table I Renal lithium clearance was significantly decreased in Groups III and VI the rats that were rehydrated by *ad libitum* water intake or by the administration of a 9 ml/h water load A normal level of lithium clearance occurred in the dehydrated rats Group II in the rats rehydrated by *ad lib* am 0 9 % NaCl intake Group IV and in the rats that were in a n rmal sta of

| Lithium clearance ml/min/100 g | Sodium clearance ml/min/100 g | Creatinine clearance ml/min/100 g | Lithium Creatinine clearance ratio |
|---|---|---|---|
| 0.11 ± 0.0[illegible] | 0.0027 ± 0.0013 | 0.65 ± 0.09 | 0.17 ± 0.0[illegible] |
| 0.12 ± 0.0[illegible] | | | |
| 0.06 ± 0.0[illegible]* | 0.000[illegible] ± 0.0001* | 0.63 ± 0.06 | 0.09 ± 0.03* |
| 0.13 ± 0.03 | 0.0[illegible]08 ± 0.0164 | 0.71 ± 0.07 | 0.18 ± 0.04 |
| 0.12 ± 0.01 | | | |
| 0.07 ± 0.01* | | | |

hydration when the administration of the 9 ml/h water load began (Group V)

No correlation was observed between the volume of urine excreted and the level of lithium clearance. The reduced lithium clearance in Group III was associated with an average urine volume that did not differ significantly from that in Group I while the normal level of lithium clearance observed in Group II was associated with an average urine volume that was significantly reduced compared to the other groups.

Compared to the control group (Group I) sodium clearance was significantly decreased in Group III and increased, although not significantly, in Group IV. No significant differences were observed in the level of creatinine clearance in Groups I, III and IV. The lithium creatinine clearance ratio in Group III was significantly lower than the ratios in Groups I and IV.

## Discussion

The main finding of this experiment is the marked decrease in renal lithium clearance in the rats during voluntary or forced rehydration with water. The fact that the lithium creatinine clearance ratio was lower in the rats rehydrated with water (Group III) than in the controls (Group I) indicates that the decreased renal lithium clearance was due to an increased fractional reabsorption of lithium in the renal tubules. The administration of 0.9 % NaCl prevented the rise in the fractional reabsorption of lithium during rehydration.

There was a relationship between the renal clearance of lithium and sodium in the rats rehydrated with water or 0.9 % NaCl. The decreased lithium clearance in the rats rehydrated with water was associated with a decreased sodium clearance while the normal level of lithium clearance observed in the rats rehydrated with 0.9 % NaCl was associated with a slight elevation in sodium clearance. This suggests that the same mechanism may be involved in the regulation of urinary sodium and lithium excretion during rehydration with water or 0.9 % NaCl.

Atherton and co-workers (Atherton *et al.* 1968 and 1970) studied the effects of water or 0.9 % NaCl administration on sodium and potassium excretion in dehydrated conscious rat. In agreement with the present results they found that the decreased urinary excretion of sodium was more pronounced when the rats we

rehydrated with water. They suggested that changes in renal haemodynamics, medullary osmolality and ADH activity may be involved in the effects of water and 0.9 % NaCl on the urinary excretion of sodium and potassium in rats. The relationship between the urinary excretion of sodium, potassium, and lithium suggests that these factors may also be involved in the regulation of renal lithium clearance in the rat during rehydration with water or 0.9 % NaCl.

The two minor findings of this experiment are that renal lithium clearance remained normal during dehydration (Group II) and also during the administration of a large water load to rats in a normal state of hydration (Group V). The latter result agrees with the observation that lithium clearance remains normal when water loads are administered to normally hydrated human subjects (Thomsen and Schou 1968).

The serum lithium concentration in the rats averaged 0.65 mEq/l. The administration of lithium in the rats' food maintained the serum lithium level in the range recommended for the treatment of psychiatric patients (Schou 1968). An implication of the present findings is related to the management of patients that become dehydrated while taking lithium. Renal lithium clearance may decrease and the serum lithium level may rise if the patients are rehydrated with water alone. The administration of sodium chloride may help maintain renal lithium clearance at a normal level during rehydration of patients taking lithium.

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Acta physiol scand 1974 90 431—437

From the Department of Zoophysiology University of Goteborg Sweden

# Drug Effects on Isolated Artery Strips from Two Teleosts, Gadus morhua and Salmo gairdneri

By

Susanne Holmgren and Stefan Nilsson

Received 5 June 1973

## Abstract

Holmgren S and S Nilsson *Drug effects on isolated artery strips from two teleosts Gadus morhua and Salmo gairdneri* Acta physiol scand 1974 *90* 431—437

The presence of adrenergic and cholinergic receptors in the celiac arteries of the cod *Gadus morhua* and the rainbow trout *Salmo gairdneri* has been investigated Affinities and intrinsic activities of different catecholamines and acetylcholine have been determined from cumulative dose response curves The $pD_2$-value for acetylcholine was 6.62 ± 0.20 in the rainbow trout arteries while the cod arteries only responded to acetylcholine in concentrations of 10 M or more In both species the potencies of the catecholamines producing contraction of the arteries were in the order adrenaline > noradrenaline > phenylephrine while isoprenaline did not contract the preparations In cod arteries competitive antagonism was obtained with yohimbine ($pA_2 = 7.3$) phentolamine ($pA_2 = 8.0$) and propranolol ($pA_2 = 6.1$) while phenoxybenzamine produced non-competitive blockade of the response to noradrenaline ($pM_{50} = 6.9$) It is concluded that although the rainbow trout celiac artery could possess double innervation it is unlikely that a cholinergic vasomotor innervation plays any role in regulating the cod celiac artery A solely adrenergic alpha receptor mediated innervation is therefore proposed for the cod celiac artery

From pharmacological experiments on isolated major arteries from trout (*Salmo trutta*) eel (*Anguilla occidentalis australis*) toad (*Bufo marinus*) and lizard (*Tiliqua rugosa*) Kirby and Burnstock (1969) suggested a mainly cholinergic vasomotor innervation in lower vertebrates An increased influence of adrenergic vasomotor fibres in the course of evolution from fish to higher vertebrates is also proposed (Kirby and Burnstock 1969 Burnstock 1969)

Adrenergic innervation of blood vessels in teleosts has been shown by fluorescent histochemistry (Leontieva 1966 Baumgarten 1967 Read and Burnstock 1968 1969 Gannon and Burnstock 1969 Kirby and Burnstock 1969 Nilsson 1973) The systemic vascular bed is constricted by catecholamines and acetylcholine (Reite 1969 Stray Pedersen 1970 Nilsson 1972 although the sensitivity to acetylcholine appears to be low in the cod *Gadus morhua* (Reite 1969 Nilsson 1972) A mainly adrenergic vasomotor innervation was therefore suggested in the gas gland of the swimbladder in this species (Nilsson 1972)

TABLE I Affinity (pD) and intrinsic activity (α) values for the different agonists tested on celiac artery strips The values are given as means ± S E Numbers within parenthesis indicate number of experiments

| | | Acetylcholine | Adrenaline | Noradrenaline | Phenylephrine |
|---|---|---|---|---|---|
| Cod | pD | < 2 (11) | 6.43 ± 0.04 (37) | 6.06 ± 0.03 (119) | 5.63 ± 0.0[illegible] (10) |
| | α | – | 1.00 | 0.94 ± 0.03 (11) | 0.41 ± 0.05 (10) |
| Rainbow trout | $pD_2$ | 6.62 ± 0.20 (11) | 7.96 ± 0.13 (37) | 7.65 ± 0.17 (8) | 5.61 ± [illegible] ([illegible]) |
| | α | 1.30 ± 0.10 (11) | 1.00 | 0.83 ± 0.11 (8) | 0.53 ± 0.07 (6) |

of both rainbow trout and cod arterses A strong relaxation could be produced by isoprenaline in cod arteries contracted by noradrenaline (Fig 3) If dose response curves were obtained for noradrenaline a parallel shift of these could be seen in the presence of isoprenaline (Fig 4)

*Effects of adrenergic antagonists on cod arteries*

Experiments with adrenergic blocking agents were carried out on cod arteries only Yohimbine (Fig 5) phentolamine and propranolol produced parallel shifts of the dose response curves for noradrenaline while phenoxybenzamine depressed the maximum response (Fig 6) $pA_2$ $pA_{10}$ (Fig 7) and $pM_{50}$ values for the blocking agents were calculated and are summarized in Table II The $pM_{50}$ value for phenoxybenzamine is determined from dose response curves obtained after 1 h exposure to the drug

## Discussion

Presence of cholinergic receptors in the rainbow trout artery is demonstrated by the present investigation although no experiments have been carried out here to

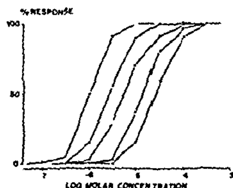



Fig 5

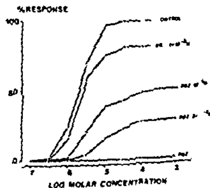



Fig 6

Fig 5 Cod celiac artery Effect of increasing doses of yohimbine on the dose response curve obtained with noradrenaline From left to right control yohimbine $10^{-7}$ M $3\times10^{-7}$ $10^{-6}$ M and $3\times10^{-6}$ M

Fig 6 Cod celiac artery Effect of increasing doses of phenoxybenzamine on the dose response curve obtained with noradrenaline

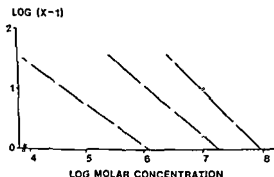



Fig 7 Cod celiac artery Calculation of pA values for competitive antagonists Each point represents an average of 3—6 runs *Legend* ● propranolol □ yohimbine O phentolamine

further determine their nature The effects of acetylcholine on the trout (*Salmo trutta*) ventral aorta was blocked by atropine suggesting that acetylcholine acts on muscarinic receptors (Kirby and Burnstock 1969)

On the cod artery acetylcholine had very small effects This could not have been due to high cholinesterase activity since the esterase resistent carbacholine and methacholine were without effects and pretreatment with eserine did not visibly incrase the response to acetylcholine In the cod therefore it is unlikely that a cholinergic innervation plays any great role in regulating the celiac artery

The adrenergic receptors mediating contraction of both rainbow trout and cod arteries appear to be of the alpha type The order of potency in this case both affinity pD and intrinsic activity $\alpha$ for the adrenergic agonists is adrenaline > noradrenaline > phenylephrine whilst isoprenaline does not contract the preparation (Ahlquist 1948 1962)

In the cod arteries the presence of adrenergic alpha receptors is further confirmed by use of alpha adrenergic blocking agents The pA values of the competitive drugs agree well with those obtained from mammalian tissues (Ignarro and Titus 1968 Kohli and Ling 1967 Guimarães 1969) Competitive blockade of the contraction produced by noradrenaline is obtained with yohimbine and phentolamine in the concentrations used and non competitive blockade is seen with phenoxybenzamine It should be noted however that the beta receptor blocking agent propranolol has pA values which are higher than those obtained from mammalian tissues In rabbit aorta for instance the $pA_{10}$ for propranolol on alpha receptors is 3 7 (Kohli and Ling 1967) Kirby and Burnstock (1969) also noted that both alpha

TABLE II Cod celiac artery pA and pM values for adrenergic antagonists

| | $pA_2$ | $pA_{10}$ | pM |
|---|---|---|---|
| Yohimbine | 7 3 | 6 1 | — |
| Phentolamine | 8 0 | 7 0 | — |
| Propranolol | 6 1 | 4 6 | — |
| Phenoxybenzamine | — | — | 6 9 |

and beta blocking agents could be used for blockade of the excitatory responses obtained with catecholamines on major arteries from several lower vertebrates

The effect of isoprenaline on the dose response curve for noradrenaline on cod arteries suggests a mainly competitive blockade of the alpha receptors rather than an effect on inhibitory beta receptors as responsible for the relaxation seen in a noradrenaline contracted preparation (Fig 4) The weak and inconsistent response to isoprenaline of arteries not previously contracted with noradrenaline give no conclusive answer to whether beta receptors mediating relaxation exist or not

It is concluded from the present results that adrenergic alpha receptors are present in the cod celiac artery and also in the rainbow trout artery although no experiments with blocking agents were performed in this species Cholinergic receptors mediate contraction of the rainbow trout artery, but the cod artery is highly insensitive to acetylcholine and thus a cholinergic innervation of the cod celiac artery seems unlikely Isoprenaline acts as a competitive antagonist on alpha receptors of the cod celiac artery but presence of inhibitory beta receptors is still not clearly shown

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*Acta physiol. scand. 1974. 90. 438—444*

From The Institute of Physiology, University of Oslo, Norway

# Sympathetic Vasodilatation, Kallikrein Release and Adrenergic Receptors in the Cat Submandibular Salivary Gland

By

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**Abstract**

GAUTVIK, K., M. KRIZ, K. LUND-LARSEN and B. A. WAALER. *Sympathetic vasodilatation, kallikrein release and adrenergic receptors in the cat submandibular salivary gland.* Acta physiol. scand. 1974. 90. 438—444.

The adrenergic receptors involved in secretion of kallikrein and the vasodilatation occurring subsequent to sympathetic nerve stimulation have been studied in the cat submandibular salivary gland. Measurements were made of kallikrein content in glandular homogenate and of venous outflow and glandular perfusion pressure. Isoprenaline failed to cause secretion of kallikrein in contrast to sympathetic nerve stimulation and close arterial injections of noradrenaline. Nerve stimulation and noradrenaline reduced the amount of kallikrein in the glands by 60 and 50 per cent, respectively. The $\alpha$ blocking agent Regitin® inhibited completely the secretion of kallikrein induced by nerve stimulation, while adrenergic $\beta$-blockade had no inhibitory effect on enzyme secretion. In this gland, therefore, the secretion of kallikrein is mediated entirely through activation of adrenergic $\alpha$ receptors.

The $\beta$ blocking agent propranolol reduced the nerve induced vasodilatation by about 40 per cent. This sympathetic vasodilator response was present, but markedly reduced after pretreatment with Regitin® which abolished kallikrein secretion and the vasoconstrictor phase of the nerve induced vascular response. This study and an earlier report (Gautvik, Kriz and Lund-Larsen 19[illegible]a) suggest that $\beta$ adrenergic receptors in the glandular vessels as well as $\alpha$-adrenergic receptors involved in the secretion of kallikrein play a role in the vasodilatation observed in this gland subsequent to sympathetic nerve stimulation.

The origin of the vasodilatation commonly seen at the end of—or immediately after—stimulation of the sympathetic nerves to the submandibular salivary gland of the cat has been subject to discussion during the years. There is a general agreement that reactive hyperaemia alone cannot account for the dilatation, as the vasodilator effect of a comparable period of circulatory arrest is much smaller (Hilton and Lewis 19[illegible]).

Carlson (1907) suggested that specific dilator nerves were responsible for the vasodilatation. [illegible] *et al.* (19[illegible]) more specifically claimed that the vasodilatation was mediated through the action of neuro-transmitter on $\beta$ adrenergic receptors in the vasculature of the gland.

Results obtained already by Barcroft and Piper (1912) however indicated that metabolites released from gland cells on sympathetic nerve stimulation were responsible for this vasodilation Hilton and Lewis (1956) suggested that the postulated chemical mediator was plasma kinins since they were able to recover glandular kallikrein an active plasma kinin forming enzyme from the perfusate after stimulation of the organ with sympathetic amines Recent studies by Gautvik *et al* (1972 a) more directly link the formation of plasma kinins to the vasodilatation induced by noradrenaline They showed that the vascular responses to close arterial injections of noradrenaline in the *in situ* perfused cat submandibular salivary gland depended on the organ s ability to form plasma kinins In glands which were perfused with solutions containing no substrate for cat salivary kallikrein and where plasma kinin formation therefore was greatly impaired the vasodilator effect of noradrenaline was abolished and a brisk vasoconstrictor response was observed instead This vasoconstriction could be reversed when the gland s ability to form plasma kinins was restored by perfusing the organ with solutions containing substrate for cat salivary kallikrein These findings however did not reveal the type of adrenergic receptors involved in release of kallikrein

From earlier studies (Ohlin 1964) it is known that adrenergic receptors of the α type dominate in all salivary glands examined and that macroscopic secretion of saliva as well as the initial vascoconstriction can be abolished by α receptor blocking agents (Emmelin 1955) However the adrenergic mechanism regulating secretion of enzymes might not be identical to that controlling transport of electrolytes and water The present study was therefore designed to evaluate the type of adrenergic receptor responsible for release of kallikrein An attempt was also made to see if there was any correlation between this release of kallikrein and the vasodilatation seen subsequent to a sympathetic stimulation The results show that adrenergic α receptors mediate the secretion of kallikrein whereas 2 different adrenergic mechanisms play a role in the sympathetic vasodilator response One does probably involve β receptors related to the glandular vessels and the other acts through the α receptors causing the secretion of kallikrein which in turn leads to formation of plasma kinins

## Materials and methods

The experiments were carried out on the cat submandibular salivary gland Cats (2 5—4 5 kg) of both sexes were anaesthetized with i p injections of pentobarbitone (30—40 mg/kg) The trachea was divided and cannulated The animals had their spontaneous respiration during the experiments The submandibular gland on both sides were isolated The experiments were carried out on one gland (stimulated gland) whereas the contralateral organ served as control The lingual artery on both sides was isolated cannulated and the *arterial blood pressure* recorded using a Statham pressure transducer (P 23 De) connected via a 700—252 DC carrier preamplifier to a multichannel recorder *Venous blood flow* was recorded by a photoelectric drop counter as described by C ... k (1970 a)

*Saliva secretion* was observed after cannulation of the salivary ducts on both sides In Fig 1 is shown a schematical drawing of the gland preparation

*Sympathetic nerve stimulation* The sympathetic nerves in the neck were dissected free and cut On the side of the stimulated gland the distal end of the nerve was connected to a platinum fluid electrode The indifferent electrode was placed on the leg The nerve fibres were stimulated supramaximally (9—14 V) with square wave pulses of 1 ms duration at 20 Hz The stim

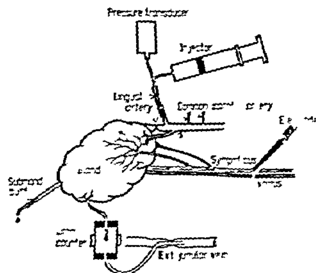



Fig. 1. Schematical drawing of preparation of the submandibular salivary gland. The arterial supply to the gland has been isolated. All branches from the common carotid artery were tied off except the lingual artery which was cannulated and used for recording of the glandular perfusion pressure. The duct and the glandular vein were cannulated. Saliva secretion was observed visually and venous flow recorded by a photoelectric drop counter before the blood was returned to the animal. Both vagi and the sympathetic nerves were cut, the peripheral end of the latter was then connected to a stimulation electrode.

ulation sequence was 15 s of stimulation with 85 s intervals. The total stimulation period usually lasted 30 min. To eliminate parasympathetic influence the vagus nerves were also cut on both sides.

*Secretion of glandular kallikrein.* The glandular kallikrein content remaining in the stimulated gland was used as an inverse measure of the degree of enzyme secretion which had taken place. The hydrolysis of α-N-Benzoyl-L-Arginine Ethyl ester (BAEE) was used for quantitative evaluation of the kallikrein activity in glandular homogenate as described by Gautvik, Kriz and *Lund-Larsen* (1972 b).

*Drugs.* Noradrenaline was obtained from Norsk Astra AS, Oslo, and Isoprenaline from the University Hospital, Oslo. Propranolol (as chloride, Inderal® ICI) and Dibenzylamine(?) methanesulfonate (Regitin® Ciba) were used.

## Results

*Stimulation and blockade of β-adrenergic receptors.* In 2 expts. isoprenaline was used as a β-adrenergic agonist and injected close arterially to the gland. Doses of 0.1 to 1 μg were given at 3 min intervals. Such doses caused vasodilatation without production of saliva (Fig. 2). After administration of a total dose of isoprenaline up to 20 μg, the glands were removed and assayed for kallikrein content. Only small changes (−12 % and −6 %) were found in the level of kallikrein activity in these 2 isoprenaline treated glands as compared to the contralateral control glands. During isoprenaline administration the venous blood was not returned to the animal.

β-adrenergic blockade was obtained by i.v. injections of propranolol (0.3 mg/kg). Prior to the blockade the smallest dose of isoprenaline which gave vasodilatation was tested out and 10 times this dose (usually 1 μg) was used to test the effectiveness of the β-blockade. In spite of a complete β-blockade the kallikrein content showed an average reduction of about 50 per cent (range 19—82 per cent) (Fig. 3) β-blocked in glands of 6 cats exposed to stimulation of the sympathetic nerves. This is not significantly different from the results obtained in a group of 7 animals which had sympathetic nerve stimulation but were not treated with propranolol. In this group

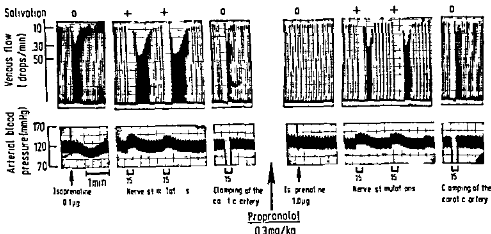



Fig 2 Typical secretory and vascular responses in a submandibular salivary gland to stimulation of the sympathetic nerves and to close arterial injections of isoprenaline before and after administration of propranolol The gland had its own blood supply and the various parameters were recorded as explained in Methods A vasoconstriction is recorded as an increased distance between drops of venous blood Note that the pressure in the lingual artery (which is identical to or very close to the glandular perfusion pressure) remained at the same level throughout the experiment

the difference in kallikrein content between the nerve stimulated gland and the contralateral unstimulated gland was on the average 61 per cent (range 19—82 per cent) (Fig 3 unblocked)

In all glands which were submitted to adrenergic $\beta$ blockade by propranolol the typical vascular reaction to sympathetic nerve stimulation was a marked vasoconstriction followed by a reduced vasodilator response In Fig 2 are shown typical secretory and vascular responses to sympathetic nerve stimulation and to isoprenaline injections before and after administration of propranolol As a result of the $\beta$ blockade the vasodilator effect of 1 0 $\mu$g isoprenaline was abolished (no increase in flow) and the nerve induced vasodilatation was reduced by about 40 per cent Furthermore the pressure in the lingual artery which in these experiments was identical to or very close to the glandular perfusion pressure did not change after administration of propranolol

*Stimulation and blockade of α-adrenergic receptors* Injection of noradrenaline caused secretory and vascular responses very similar to those induced by sympathetic nerve stimulation (Gautvik *et al* 1972 a) Repeated close arterial injections of noradrenaline reduced kallikrein activity in the gland by about 50 per cent (Gautvik *et al* 1972 a) This is the same degree of enzyme reduction as presently observed after repeated sympathetic nerve stimulation (Fig 3 unblocked)

Blocking of the $\alpha$ adrenergic receptors was performed by i v administration of Regitin 5 mg/kg This concentration inhibited completely the secretion of kallikrein normally provoked by sympathetic nerve stimulation (Fig 3 $\alpha$ blocked) In 7 $\alpha$

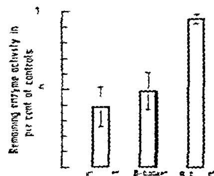


Fig. 3. Remaining kallikrein activity in sympathetically stimulated submandibular salivary glands without any medication (unblocked), after administration of propranolol in doses of 0.3 mg/kg (β blocked) and after administration of Regitin in doses of 5 mg/kg (α blocked). The kallikrein activity in each of the contralateral nonstimulated control glands was set to 100 per cent, and the enzyme activity in the individual stimulated glands calculated accordingly. Mean values ±S. E. are shown for groups of 7, 6 and 7 animals respectively. The kallikrein activity was measured as described in Methods and the actual enzyme activity (μmol BAEE hydrolyzed 1 g tissue × min) in the various control groups was: Unblocked (4 animals) mean 34.6, range 14.6—65.0. β blocked (7 animals) mean 62.7, range 37.0—114.2. α blocked (7 animals) mean 125.? range 66.6—169.0.

adrenergic blocked glands about 9? per cent of the kallikrein activity was recovered after a total stimulation period of 30 min. Also macroscopic secretion of saliva was abolished in 6 of the animals. The seventh had a very scanty spontaneous secretion which was not correlated to the periods of nerve stimulation.

Regitin in the concentration used abolished the vasoconstrictor response to sympathetic nerve stimulation (Fig. 4) as well as to close arterial injection of noradrenaline (data not shown). These findings confirm earlier results of Emmelin (19??). In addition ... as well as close arterial administration of Regitin caused a reduction in both glandular perfusion pressure and in resting venous blood flow (Fig. 4). As is also seen in Fig. 4, the post-stimulatory sympathetic vasodilator response is still present but markedly reduced in such an α blocked gland. However, with the reduced venous flow and perfusion pressure this finding must be interpreted with caution. If one postulates that the lingual arterial pressure is identical to the gland's ... perfusion pressure, calculations which take into account the altered basal ... that the remaining vasodilator response after α blockade amounts to about half of that observed prior to Regitin administration.

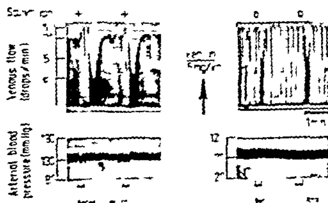



Fig. 4. Typical secretory and vascular responses in a ... gland ... salivary gland ... stimulation of the sympathetic nerves before and after ... minute ... of Regitin. For further details see Methods. Note that Regitin administration was followed by reduction of the pressure in the lingual artery and also in the venous flow.

## Discussion

The secretion of kallikrein in the cat submandibular salivary gland has been shown to be mediated via α adrenergic receptors This conclusion is based upon the following data 1) Noradrenaline (Gautvik *et al* 1972 a) but not isoprenaline caused secretion of kallikrein 2) Adrenergic α blockade by Regitin inhibited completely the secretion of kallikrein normally elicited by smpathetic nerve stimulation while adrenergic β blockade by propranolol had no such effect In addition we conclude that secretion of kallikrein and of water and electrolytes are influenced through the same type of adrenergic mechanisms

Since the secretion of kallikrein was thus mediated through α adrenergic receptors it was possible to assess directly the role of plasma kinins in the sympathetic vasodilatation which follows subsequent to sympathetic stimulation In a β blocked gland where the vasodilator effect of isoprenaline was abolished a major component of the nerve induced vasodilatation remained Very little is known about the interaction between pla ma kinins and their vascular receptors However it has been shown earlier that the vasodilator effect of bradykinin is not significantly altered in a propranolol blocked gland (Gautvik 1970 b) β blockade did however reduce the vasodilator response to sympathetic nerve stimulation and this effect cannot be due to a diminished arterial inflow since propranolol administration did not change significantly the glandular perfusion pressure On the other hand an α blocked and kallikrein blocked gland , which showed no vasoconstriction on nerve stimulation or after noradrenaline injections also exhibited a marked reduction in the nerve rduced vasodilatation Evaluation of the relative importance of the influence mediated via α and β adrenergic receptors in this connection met with some difficulties Thus administration of Regitin both locally and i v changed the whole experimental background by reducing the perfusion pressure and the venous outflow from the gland However the observation that the adrenergic after vasodilatation was not completely abolished by either α or β blockade strongly suggests that both α and β adrenergic receptors are involved in mechanisms underlying this vascular reaction

Since the liberation of kallikrein is shown to be mediated only via α receptors the vasodilatation resulting from activation of the β receptors may be entirely due to direct effects on vessel walls This is in accordance with the view of Carlson (1907) and of Bhoola *et al* (1965) *The contribution of the α receptors to the adrenergic* vasodilatation is probably via release of kallikrein and subsequent plasma kinin formation Our data are thus al o in agreement with the theory of a chemical mediation of the sympathetic after vasodilatation as proposed by Barcroft and Piper (1912) and by Hilton and Lewis (1956) Held together the results of this study and of those of a previous work (Gautvik *et al* 1972 a) strongly suggest that the sympathetic after vasodilatation in the cat submandibular gland is mediated through 2 different mechanisms Firstly there is a probable direct nervous influence on vessels acting via β receptors Secondly there is a chemical mechanism involved where activation of α receptors cau e relea e of the plasma kinin forming enzyme kallikrein which will again give formation of kinins in the gland

## Acknowledgements

This work has been supported, through grants to The Institute of Physiology, from the Norwegian Research Council on Science and the Humanities and from The Nansen Foundation. This support is gratefully acknowledged.

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Acta physiol scand 1974 90 445—450

From the Laboratory for the Theory of Gymnastics University of Copenhagen Denmark

# Actions of Intravenous $Ca^{++}$ and $Na^{+}$ on Body Temperature in Rabbits

By

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## Abstract

NIELSEN B *Actions of intravenous $Ca^{++}$ and Na on body temperature in rabbits* Acta physiol scand 1974 *90* 445—450

Rectal temperatures in rabbits were found to increase when hyperosmotic NaCl solution were infused in the marginal earvein while infusion of CaCl caused a fall in rectal temperature The latter effect was much more pronounced in cold (14 $T_a$) environment It could be shown that the ions affected the peripheral circulation and therefore the heat loss It is concluded that the observed changes in body temperature most likely are secondary to the peripheral changes in heat loss and not a thermoregulatory change in the set point of the hypothalamic centres

Experiments by Feldberg Myers Cooper (ref 2—5) and their co workers give support to the theory that the chemical composition of the cerebrospinal fluid (CSF) in the 3d ventricle adjacent to the temperature centres in the hypothalamus exerts an influence on the body temperature Feldberg Myers and Veale (1970) Myers and Veale (1971) and Myers Veale and Yaksh (1971) postulate that the set point of the thermoregulatory system is determined by the balance between sodium and calcium in CSF Changes in the Na /Ca ratio produced by perfusion of posterior hypothalamic sites brought about changes in the level around which the body temperature was regulated

Experiments with human subjects (B Nielsen *et al* 1971 Snellen *et al* 1972 a b) demonstrated that body temperature in human subjects during rest and exercise is influenced by the osmolarity of the blood Decreases in plasma osmolarity produced by drinking water decreased body temperature while increases in plasma osmolarity produced by drinking 3 % NaCl solutions or by dehydration caused an increase in body temperature to a higher than normal level Later it was found that osmotic changes in the plasma produced by drinking CaCl solutions caused a fall in body temperature while NaCl caused a rise (B Nielsen to be published)

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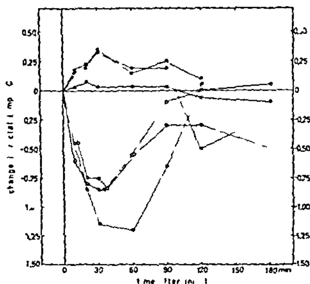



Fig ? Change in rectal temperature following injection of 20 ml aCl solution ● or 20 ml $CaCl_2$ solution O at 14 C environmental temperature 6 expts on 3 animals

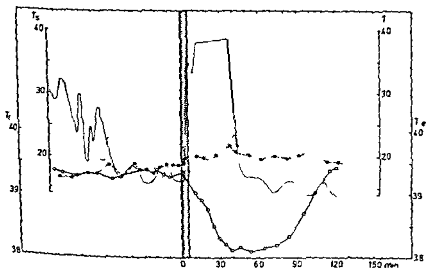



Fig 3 Time course of rectal temperature and earskin t r perature before and after injection of 20 ml Na or $Ca^{++}$ solution at 14 C T One animal Time of injection is shown by the shaded area. T {Na ● / Ca O} T {Na / $Ca^{++}$ ——}

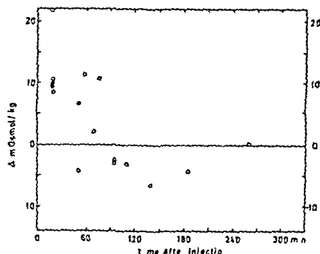



Fig 4 Change from preinfusion values in plasma osmolarity in blood samples taken at various times after infusion of sodium ● or calcium O solutions

## Results

The results from 9 expts at 24° C with Na and 9 expts with Ca infusions in 3 rabbits are hown in Fig 1 It is seen that NaCl caused a rise in body temperature lasting for more than 3 h while CaCl produced an initial fall in body temperature followed by a gradual return to the pre infusion level

At low ambient temperature (14° C Fig 2) the temperature fall produced by Ca was much more pronounced than at 24° C while the effect of Na was of the same order of magnitude about $+\frac{1}{4}-\frac{1}{3}$° C) as at 24° $T_a$

In Fig 3 the effect of the injections of 20 ml Na or Ca solutions on T and T at 14 C are shown Ca produced a marked vasodilatation immediately after the injection while Na caused the ear vessels to constrict as demonstrated by the fall in skin temperature

Fig 4 hows the changes in osmolarity due to the injection at different times after the injection Plasma [Na ] and [$Ca_t^{++}$] was measured before and 15 min after injection of both Na and Ca solutions in 3 animals The average increase in [Na] was − 6 mEq l − 4 3 % ) and in [$Ca_{tot}$ ]+ 6 3 mEq/l ( + 85 %)

## Discussion

The result of these experiments clearly demonstrate an effect of the ionic composition of the blood on body temperature It is also obvious that the effect is due to a specific action of the Na and/or Ca ions and not to the effect of the changed osmolarity since the changes in plasma osmolarity were of the same order of magnitude—about 10 O mol kg in the first hour after the injection—with both Ca and Na-chloride solution Fig 4 The activity level of the animals was not different before and after injections neither with Na nor Ca as judged by the "activity recordings

There is good evidence for the assumption that the mechanism by which body temperature is changed acts via the peripheral vasomotor tone and heat loss

Nevertheless it is not clear where Na and $Ca^{++}$ ions exert their effect The hypothesis that the ions act directly on the set point of the hypothalamic centres can be doubted since the observed vasomotor changes occur within the first 2—5 min after the injections while the passing of ions especially divalent ions from blood to brain takes a considerable time (hours) Levin and Patlac 1972 Bradbury *et al* 1972

But several areas in the central nervous system have been shown to have less restrictive blood brain barriers Therefore the possibility of a central action for the ions cannot be ruled out completely

Their action could be on osmoreceptors in the brain In recent experiments K Olsson (1972) found that intracarotid infusions of hypertonic NaCl in the goat produced greater effects than equiosmolal hypertonic solutions of fructose glucose urea glycerol etc indicating that the osmoreceptors responsible for water intake and ADH secretion are specific Na receptors placed near the 3d ventricle and exposed to Na in the CSF

An alternative explanation involves a peripheral effect of Ca and Na either on the vascular smooth musculature or perhaps by blocking the neuromuscular transmission in the sympathetic vasoconstrictor system or stimulating a vasodilator system to the ear vessels

Increased osmolarity has been shown to cau e decreased mechanical and electrical activity in vascular smooth muscle *in vitro* and *in vivo* (rats humans) This effect was ascribed to osmotic shrinkage of the tissues (Mellander *et al* 1967 Johansson and Jonsson 1968 Lundvall *et al* 1969)

On the other hand Ca in general have an excitatory effect on synapses and transmitter release (*e g* Richard and Sercombe 1971 Stjarne 1973 Llinas *et al* 1972)

It may be mentioned that Ca injections also always caused an emptying of the bladder in the animals

In other words the actions of the ions observed in the present experiments are not easily explained by known effects of Ca and/or osmolarity changes

The difference in magnitude of the reaction to Ca ions at 14 compared to at 24° C must be explained by the greater effect of an evoked vasodilation at the lower ambient temperature

Although no definite conclusion can be drawn it is most likely that the alterations in the cutaneous vascular tone which undoubtedly underly the observed effects of $Ca^{++}$ and Na on body temperature are due to a peripheral action of the ions rather than to a cerebral action involving a change in set point of the hypothalamic centres

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Acta physiol scand 1974 90 451—461
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# Contraction in Venous Smooth Muscle Induced by Hypertonicity Calcium Dependence and Mechanical Characteristics

By

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## Abstract

ANDERSSON C P HELLSTRAND B JOHANSSON and A RINGBERG *Contraction in venous smooth muscle induced by hypertonicity Calcium dependence and mechanical characteristics* Acta physiol scand 1974 *90* 451—461

A tonic increase in tension was observed in the isolated rat portal vein in response to large increases in extracellular tonicity (1 5—3 times normal) The purpose of this study was to find out whether this tension increase was due to active contraction or passive shrinkage The metabolic dependence of the response and its mechanical characteristics as revealed by quick release experiments lead to the conclusion that active contraction is the predominant mechanism The maximal force developed in the hypertonicity contracture amounted to 31 per cent of the maximal K contracture obtained in isotonic medium The hypertonicity contracture occurred independent of the level of membrane potential and in the absence of external $Ca^{++}$ (below $10^{-9}$ M) It is thus elicited through mechanisms which differ in these respects from the electromechanical coupling operating in the K contracture The speed of shortening against a given afterload was found to be lower in a hypertonicity contracture than in a K contracture of comparable isometric force Explanations for observed differences between the two types of contracture are discussed

Earlier studies have shown that moderate degrees of hyperosmolality inhibit the spontaneous myogenic activity of the rat portal vein by reducing the frequency of phasic contractions (Johansson and Jonsson 1968) If the external medium is made strongly hypertonic phasic activity can be completely abolished but the muscle then develops a tonic increase in tension (see Fig 1 below) The question arose as to whether this tonic response is due to active contraction or only to passive shrinkage of the tissue The purpose of the present study has been to elucidate this question

A preliminary report on some of these results have been presented previously (Andersson *et al* 1972)




Fig 1 Effects of hyperosmolality on myogenic activity and isometric force of rat portal vein in normal ionic environment

## Methods

Rats of the Sprague Dawley strain with body weights between 250 and 350 g were used The animals were killed by cervical fracture and segments of the portal vein 4—7 mm long were dissected out

In *experiments with isometric recording* the muscles were mounted in a 50 ml thermoregulated organ bath One end of the preparation was fixed to a muscle holder and the other end connected to a force transducer (Grass FT03) The passive force applied was 200 to 400 dyn Recording was done on a Grass Polygraph The muscles were allowed to accommodate for at least 45 min in a modified Krebs solution of the following composition in mM NaCl 170 KCl 6 0 $MgCl_2$ 1 2 $CaCl_2$ 2 5 glucose 11 5 and tris (hydroxymethyl) amino methane (Trizma Base Sigma Chemical Company) 35 This solution had been titrated with HCl to a pH of 7 4 at 37 C or 10 C respectively the two temperatures used for this study It will be referred to below as normal solution Depolarizing K high solution was prepared by replacing 100 mM NaCl of the normal medium with equimolar amounts of KCl Nominally Ca free media were obtained by omitting $CaCl_2$ from the solutions A reduction of [Ca ] to very low levels below $10^{-9}$ M (see Hubbard Jones and Landau 1968) was achieved by addition of 1 0 mM EGTA (Sigma Chemical Company) Hyperosmolality was produced by adding sucrose urea malonamide or NaCl to the solutions described above

*Quick release experiments* were performed by using the procedures and the apparatus described by Johansson (1973) In principle the muscle was mounted horizontally between an isometric force transducer and an isotonic lever The latter could be clamped or released by an electromagnet system for studying isometric force or shortening under a constant load respectively Shortening could also be interrupted after release of the lever so that the effect of a small step change in length could be examined The resting tension was set at 50—75 dyn in these experiments in order to reduce the influence of parallel elasticity Length and force of the preparation were recorded on the Grass Polygraph The shortening velocity was determined by drawing a tangent to the length registration 100 ms after release and making a geometrical correction for the fact that the recording pens write along the arc of a circle For further details see Johansson (1973)

## Results

*1 Description of the tension response to increased osmolality*

Fig 1 shows the spontaneous isometric contractions of isolated rat portal vein in standard Krebs solution and the response to hyperosmolality produced by the addition of sucrose to a concentration of 250 mM This increase in osmolality abolished the phasic contractions but caused on the other hand a tonic increase in tension of about 200 dyn

If the tonic response is related to shrinkage of the smooth muscle cells it should appear only when hyperosmolality is produced by non permeant substances The permeability characteristics of the smooth muscle cells of the rat portal vein have

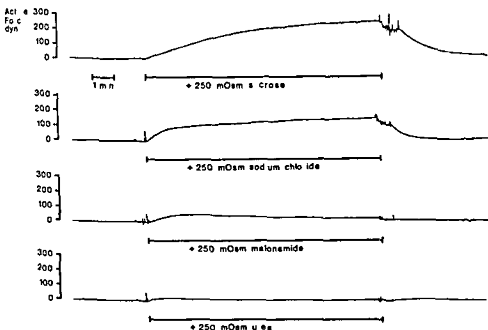



Fig 2 Isomtric tension responses of portal vein to extracellular hyperosmolality induced by various substances in nominally Ca free solution

been examined previously and information from such studies form the background of the experiment shown in Fig 2 Here the phasic activity was first abolished by omitting $Ca^{2}$ in the bath fluid a procedure which does not eliminate the tonic response to hyperosmolality (see further below) It can be seen from Fig 2 that substances like sucrose and NaCl which exert a maintained osmotic effect on the cells (Johansson and Jonsson 1968) produced a sustained tension increase whereas malonamide which slowly penetrates the cell membrane (Johansson 1970) only produced a moderate and transient response Urea which rapidly passes into the cells (Arwill Johansson and Jonsson 1969) caused just a slight and transient change Thus in order to achieve the sustained increase in force extracellular hyper osmolality must evidently be induced by substances of low permeability

The relative magnitude of the tonic increase in force as a function of extracellular tonicity is illustrated by Fig 3 Maximal response to hyperosmolality was produced by 400 mM sucrose and this effect was on the average 31 % of the active tension obtained in K contractures with 16 mM Ca in the bath (cf Hellstrand Johansson and Ringberg 1972)

The following experiments were designed with the purpose of investigating whether the response to hypertonicity is a direct effect of tissue shrinkage or whether it also involves active contraction The response to addition of 250 mM sucrose was tested with regard to its dependence on the ionic environment and metabolic supply

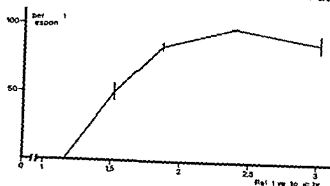


Fig 3 Relative tension increase (mean ± S E n = 4) in response to different degrees of hypertonicity produced by addition of sucrose Tonicity expressed in relation to that of normal solution

Fig 4 Ca-dependence of spontaneous activity and responses to hypertonicity, K-depolarization and noradrenalin Note that spontaneous activity, K-contracture and noradrenaline response are abolished in the Ca free medium the response to hypertonicity persists under these conditions both in the polarized and depolarized state

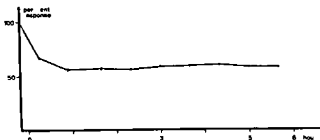



Fig 5 Relative magnitude of tension response to hypertonicity (mean ± S E n = 4) as a function of time in Ca free solution containing 1 0 mM EGTA Responses expressed in per cent of control response in normal solution containing 2 5 mM Ca⁺⁺

and with regard to its mechanical characteristics Contractures elicited by K high solution containing graded concentrations of CaCl were used as reference responses

*B Ionic dependence*

The Ca dependence of the tension increases induced by hyperosmolality K high solution and noradrenaline respectively is illustrated in Fig 4 Records A and B show that the spontaneous activity and the responses to K depolarization and noradrenaline are rapidly abolished in Ca free medium The response to hypertonicity on the other hand is maintained under these condition as shown by record C It can also be obtained in K high solution (record D) suggesting its independence of membrane potential

A closer examination of the Ca dependence of the hypertonicity response was performed in experiments where the [Ca ] was reduced below $10^{-9}$ M by addition of 1 mM EGTA At 30 min intervals the hypertonic solution was introduced for 10 min and the tension increase was recorded The results are summarized in Fig 5 Responses were expressed as a percentage of a control response obtained in normal solution with 2 5 mM Ca at the beginning of the experiment It can be seen that after an initial drop complete within the first hour the tension increase in response to hypertonicity was markedly resistant to several hours of Ca depletion Should the remaining response to hypertonicity represent an active contraction it is evidently either completely independent of Ca or capable of utilizing intracellular Ca stores which are not accessible to stimulation by K depolarization or noradrenaline

*C Metabolic dependence*

The effects of metabolic interventions on the response to hypertonicity and on the K-contracture were compared Two portal vein preparations were examined simultaneously but in different baths one of them being stimulated by hypertonicity the other by K high solution Control responses of approximately the same magnitude were produced in the two preparations by adjusting the Ca concentration of the K high medium Fig 6A shows the influence of low temperature The hyperosmolality response is almost absent at 10° C whereas the K-contracture is reduced to about 20 per cent of control In a glucose free medium bubbled with N the tension increase produced by both K and hypertonocity are diminished the K contracture to the greatest extent (Fig 6B)

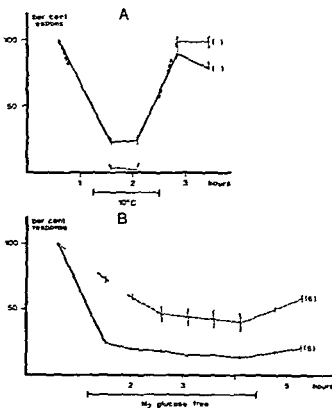



Fig. 6. Effects of reduced temperature (*A*) and of glucose and oxygen deprivation (*B*) on responses to hypertonicity (broken line) and to K depolarization (solid line). Responses expressed in per cent (mean ± S.E.) of a control response obtained after 45 min in $Ca^{2+}$ free standard solution at 37 C. Figures in parentheses represent number of muscles studied.

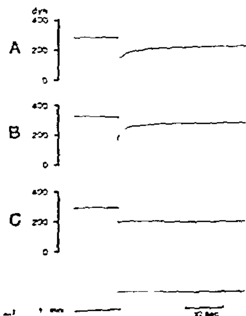



Fig. Effects on force of a small step change in length. *A*: Portal vein in medium containing 200 mM sucrose. *B*: Preparation in K-rich medium with Ca [illegible] adjusted to give about the same isometric force as in A. *C*: Preparation in normally $Ca^{2+}$ free solution but pa[illegible] stretched to [illegible] the same force as in A and B. Note pronounced recovery of force after the shortening step in A and B.

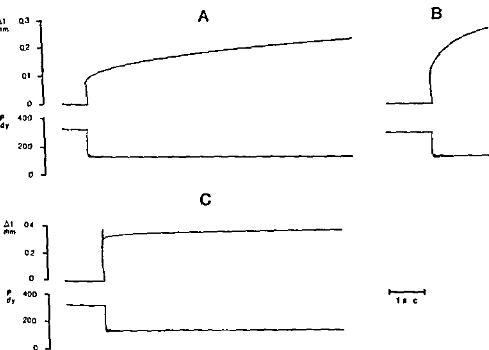



Fig 8 Isotonic quick release experiment on portal vein Upper tracing length change of preparation lower tracing isometric force A Preparation in Ca free hypertonic medium containing 250 mM sucrose *B* Same preparation in K high solution with Ca adjusted to give the same isometric force as in A *C* Same preparation in nominally Ca free solution but passively stretched to the same force as in A and B Note in A and B the progressive isotonic shortening following the immediate recoil after release

The results of these experiments suggest that the tension increase produced by hypertonicity is an active response requiring metabolic energy Another interpretation would be that the metabolic interventions might increase membrane permeability to the extent that sucrose no longer exerts its osmotics effect on the cells Jonsson's (1969) observation of an essentially unchanged [14]C sucrose space in portal veins at 1° C speaks against this alternative explanation of the results in Fig 6 A

*D Mechanical behaviour*

The change in force of an active muscle in response to a step change in length differs qualitatively from that of a resting muscle Correspondingly different length responses to a step change in force are obtained in the two situations Quick release experiments of these two kinds were performed on portal vein preparations in which the hypertonicity response was compared to the active K contracture on the one hand and to the resting state on the other

Fig 7 shows the responses of a portal vein to a step change in length In A the isometric force had been increased from the preload level of 60 dyn to about 280 dyn by exposure of the muscle to hypertonic solution An isometric contracture force

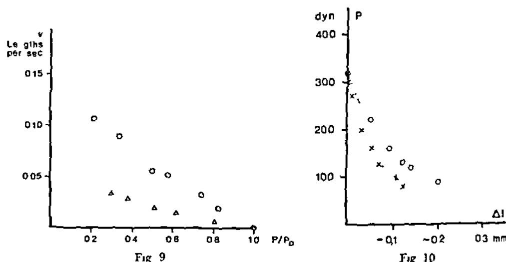



Fig 9

Fig 10

Fig 9 Force velocity relations of portal vein *Triangles* muscle in hypertonic solution Isometric force 300 dyn *Circles* same muscle in K high solution with Ca adjusted to give the same isometric force

Fig 10 Initial recoil (abscissa) on release from an isometric force of 320 dyn to different afterloads (ordinate) *Crosses* Portal vein in medium containing 250 mM sucrose *Circles* Preparation in K high medium with Ca adjusted to give an isometric tension of 3 0 dyn. Note difference in load extension curves indicating greater stiffness of series-elastic element in the hypertonic solution

of about 320 dyn was obtained in response to K high solution (B) In C the total force had been increased to a comparable level by stretching the muscle passively The immediate drop in force associated with the length step is followed in records A and B by a gradual recovery Practically no such recovery is seen in record C where the muscle behaves more like a purely elastic body

The reverse type of experiment is illustrated in Fig 8 The isometric force had been increased to about 320 dyn in records A B and C by the same procedures as in the corresponding records of the previous figure The load on the muscle was suddenly released to a constant afterload of 120 dyn The length change in records A and B shows two distinct phases (i) a fast recoil immediately on release and (ii) a much slower second phase sharply separated from the first phase by a few low amplitude oscillations due to inertia of the lever and influenced by the stiffness of the preparation The fast phase of such recordings have been interpreted to represent recoil of a passive elastic element coupled in series with the contractile element whereas the second phase has been attributed to active shortening of the contractile element itself (Jewell and Wilkie 1958) In the passively stretched preparation (C) the elastic recoil is much larger the inertial oscillations are more prominent and the second shortening phase is almost lacking This response thus consists of the recoil of a highly distensible elastic element (parallel elasticity) followed by a small creep After the first sec the muscle length stays practically constant The small creep phase might be attributed to viscous damping in part of the

parallel elasticity leading to uneven force distribution in the first sec after the release The details of this response cannot at present be interpreted in terms of smooth muscle structure

With regard to mechanical behaviour the muscle in hypertonic solution shows the qualitative characteristics of the K-contracture but differs markedly from the resting state (Fig 7 and 8) These observations lend support to the notion that the tension increase produced by hypertonicity involves active contraction Quick release experiments on hypertonicity contractures elicited after 3 h in Ca free solution with 1 mM EGTA and in glucose free solution dubbled with N respectively also showed these qualitative characteristics of active contraction This indicates that the responses remaining after 3 h under these conditions as illustrated in Fig 5 and 6 B still contain an appreciable active component

Certain quantitative differences between the contractures elicited by hypertonicity and K are apparent from Fig 8 It is seen that the second phase of shortening in record A occurs at a much slower rate than the corresponding phase of record B By releasing the muscle to different afterloads and measuring the shortening velocity of the contractile element the force velocity relation can be established Fig 9 shows the shortening velocity in muscle lengths per second as a function of force expressed as $P/P_0$ where $P_0$ is the isometric force before release In this particular experiment $P_0$ was 300 dyn in both contractures For all values of relative load the shortening velocities obtained in the hypertonicity contracture were lower than those found in the K contracture This has been a consistent finding in all of the experiments

Records A and B of Fig 8 also show a slight difference in the extent of the elastic recoil associated with the release This would suggest a difference in the stiffness of the series elastic element The load extension curve of the series elasticity can be studied by measuring the initial fast length change when the muscle is released from a contracture to different afterloads In Fig 10 are shown on the abscissa the resulting length changes when a portal vein preparation was released from an initial isometric tension of 320 dyn to the afterloads indicated on the ordinate Crosses refer to the hypertonicity and circles to the K-contracture It is seen that the muscle responds to given changes of load with smaller length steps when it is exposed to the hyperosmotic solution This indication of a greater stiffness of the series elastic element in the hypertonic medium was found in all of 6 expts of this type

## Discussion

The starting point for this study was the observation of a tonic increase in tension in the isolated portal vein on exposure to strongly hypertonic solution A similar effect has been reported earlier for dog aorta by Yamabayashi and Hamilton (1959) and for rabbit and canine pulmonary artery by Somlyo and Somlyo (1970 p 311) but the nature of the response has not been elucidated The purpose of the present study was to analyse the mechanism behind this phenomenon in portal vein with regard to the relative importance of active contraction and passive shrinkage

The results presented in sections *C* and *D* above lead to the conclusion that active

*contraction* is a predominant component of the tension increase Mechanical characteristics of active contraction remain even after the isometric response has been reduced by ionic (Fig 5) or metabolic (Fig 6 B) interventions The presence of a small passive component in the isometric response directly related to cell shrinkage cannot be excluded, but the experiments with cooling (Fig 6 A) indicate that this mechanism contributes very little to the total tension increase produced by hypertonicity Recent experiments have shown a contractile response also in frog skeletal muscle in hypertonic solutions (Gordon and Godt 1970, Lannergren and Noth 1973) These studies showed that the mechanical response consists of two components a small maintained tension increase and a larger phasic one The phasic response was found by Lannergren and Noth (1973) to resemble the caffeine contracture in its independence of membrane potential its relative resistance to changes in [Ca] and its sensitivity to tetracain The hypertonicity response of the portal vein also occurred regardless of the level of membrane potential and external Ca but further comparisons with the findings of Lannergren and Noth are difficult since the portal vein is not contracted by caffeine It is possible anyway that the smooth muscle contracture produced by hypertonicity is related to the phasic component of the skeletal muscle response the mere difference in time course does not exclude such a relationship in view of the fact that the K contracture is also phasic in the striated muscle and sustained in the portal vein

The hypertonicity contracture and the K contracture of the portal vein differ from one another in many respects Most conspicuous is the difference in the dependence on external Ca Hypertonicity might activate the contractile system of the portal vein by releasing calcium from intracellular stores in analogy with the mechanism proposed for striated muscle by Lannergren and Noth (1973) A sparse sarcotubular system which takes up divalent cations has been demonstrated in the *smooth muscle cells of portal mesenteric veins (Somlyo and Somlyo* 1971 Devine Somlyo and Somlyo 1972) The alternative explanation for the marked resistance of the hypertonicity contracture to calcium depletion would be a direct activating effect of the increased intracellular ionic strength on the contractile machinery However to our knowledge such a mechanism has not been reported for isolated actomyosin systems The resistance of the hypertonicity response to calcium depletion can be further elucidated only by direct studies of Ca content and Ca kinetics in the muscle under the actual conditions

Our studies indicate that the hypertonicity contracture is characterized by a lower shortening velocity than that of the K contracture *Increase of ionic strength* in isolated actomyosin systems including those prepared from vascular smooth muscle has been shown to give a diminished ATPase activity (Bowen and Kerwin 195[illegible] Maxwell Bohr and Murphy 1971) In view of the relation between shortening velocity and ATPase activity in different muscle preparations (see e g *Ruegg* 19[illegible]1) the lower shortening velocity of the portal vein in hypertonic solutions could be the effect of a reduced ATPase activity Attention should also be paid to the possibility of an increased internal load due to higher intracellular viscosity in the shrunken

tissue This would tend to give too low an estimate of the shortening velocity at zero external load These mechanisms have also been considered as an explanation of the low shortening velocity of skeletal muscle in hypertonic environment (Howarth 1958 Podolsky and Sugi 1967) It is intresting that the filamentary structure of smooth muscle as seen by electron microscopy changes when the cell volume is altered (Jones Somlyo and Somlyo 1973) However it is not yet clear to what extent these changes may occur in the living cell before fixation and their possible relation to our functional findings cannot therefore be judged at present

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Acta physiol scand 1974 90 462—474

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# Renal Control of Salt and Fluid Homeostasis during i v Saline Infusion

By

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**Abstract**

APERIA A and O BROBERGER *Renal control of salt and fluid homeostasis during i v saline infusion* Acta physiol scand 1974 *90* 462—474

The renal effect of infusion of hypo- iso- and hypertonic saline has been studied in dogs The infusion rate was comparatively low 1 % of the b wt./45 min The kidneys responded almost instantaneously to the infusion with diuresis and natriuresis The response was due primarily to an adaptive inhibition of tubular reabsorption Increases in filtered load of Na occurred only as a function of increased serum Na concentration *i e* only when the hypertonic saline solution were used The inhibition of tubular reabsorption was mainly signalled by the fall in serum protein concentration Changes in Na balance and serum Na concentration did not contribute to the adaptive inhibition of tubular reabsorption The effect of the diuretic and natriuretic response on fluid and Na balance depended primarily on the tonicity of the infused fluid When the 75 and 150 mEq solutions were used fluid balance became positive but did not increase much when more than 2 % of the b wt had been given When the hypertonic saline solutions were used total fluid balance became increasingly negative *The Na balance was best controlled with the hypotonic saline solution When hypertonic saline was* given Na balance increased almost at the same rate as Na was given

It is well established that expansion of the extracellular space results in increased urine flow (de Wardener *et al* 1961 Rector *et al* 1964 Earley *et al* 1964) The nature of the diuretic response has been the subject of numerous investigations This has resulted in a better understanding of the factors governing tubular sodium reabsorption The importance of intrarenal physical forces has been appreciated (Martino and Earley 1967 Brenner *et al* 1971) Several other factors have also been considered Those include changes in tubular geometry (Gertz *et al* 1965 Rector *et al* 1967) redistribution of nephron function (Jamison and Lacey 1971) and the possible existence of a natriuretic hormone (Rector *et al* 1968) So far however none of those factors seems to be of any major importance (Burg and Orloff 1968

*Keywords* Saline infusion sodium balance fluid balance tubular Na reabsorption serum protein concentration

Morgan and Berliner 1969 Bartoli and Earley 1971 Baines 1973 Wright *et al* 1969) Most studies have used micropuncture techniques and have been carried out under rather extreme physiological conditions the expansion generally exceeding 5 % of the b wt As a consequence of this relatively little information has been obtained on the usefulness of the expansion induced natriuresis for the maintenance of salt and fluid balance

In the present study conventional clearance techniques have been applied to evaluate the natriuretic response during i v fluid loads with varying salt concentration The infusion rate correspondended roughly to that used in clinical practice Information has been obtained both on the nature of the total renal response and of the resulting effect on salt and fluid balance

## Material and Methods

The experiments reported were carried out on 23 adult mongrel dogs of either sex ranging in weight from 11 to 18 kg During the week prior to the study the animals were given a diet containing approximately 65 mEq Na /day 18 h prior to the study fluid and water were removed from the animals The animals were anesthetized initially with penthotal sodium 25 mg/kg The anesthesia was maintained with nembutal in small supplements as required The dogs were intubated and kept on artificial respiration by means of an Engstrom respirator supplied with air In some of the dogs the acid base balance was repeatedly checked with an Astrup pH meter (Radiometer Denmark) The body temperatures of the dogs were kept constant at 37 C by means of rubber mattress thermostats surrounding their bodies

For infusion and pressure recording purposes exposure was made of both femoral arteries and veins left brachial vein and right carotid artery After a suprapubic incision both ureters were catheterized with infant feeding tubes size Fr 5 with ID 1 mm OD 1 3 mm (Meredith Plastics) 60 min before the experiment was started the animals were given vasopressin (*Lypressin* Sandoz) 0 75 IU/kg b wt. i v The prime dose was followed by continuous infusion of vasopressin 0 20 U/kg BW/h This was found to be the highest dosage of vasopressin that did not depress the glomerular filtration rate or raise the arterial blood pressure

Standard clearance techniques were used This included continuous infusion of inulin (Laevosan Gesellschaft Linz) and paraminohippuric acid (MSD West Point) Arterial blood samples were withdrawn at the midpoint of each urine collection period Urine was collected from both ureters during as a rule 15 min periods The arterial blood pressure was continuously recorded with a Sanborn pressure transducer

When urine had been collected for 2 control periods the i v infusion of saline was started The infusion was given into a cannula that had been inserted into the inferior vena cava *via* the femoral vein The saline solution contained either 75 150 300 and 600 mEq NaCl The infusion rate when the 75 150 and 600 mEq NaCl solutions were used was 0 202 *ml/kg/min* Thereby 1 % of the b wt. was given every 45 min When the 300 mEq NaCl solution was used the infusion rate was adjusted so that the fluid balance would not become negative When 1 % of the b wt. had been given the infusion rate equalled the urine flow of the preceeding period The studies were generally interrupted when 30 ml/kg b wt had been given

*Analytical Methods* Inulin in serum and urine was analyzed with the anthron method described by Hilger *et al* (1958) PAH was analyzed according to the method of Smith *et al* (1945) Na in serum and urine was analyzed with a flame photometer Analysis of the serum total protein concentration in 100 μl samples was made with a refractometric method (Zeiss refractometer) The hematocrit of heparinized arterial blood samples were determined by centrifuging them in glass capillaries at 10 000 RPM for 5 min

*Calculations* Reabsorbed Na is calculated as the difference between filtered and excreted Na The filtration fraction is calculated as CInulin/CPAH For statistical analysis the Student s T test has been used

TABLE I A General effects of infusing 75 mEq Na olution 1 2 and 3 of the body weight

| | Control m±1 S D | 1 % expansion | 2 % expansion | 3 expansion |
|---|---|---|---|---|
| GFR ml/min<br>Difference of mean versus control | 27 6±4 0 | +0 7±1 6<br>p < 0 2 | −0 7±3 1<br>p < 0 5 | +1 3±2 0<br>p < 0 ? |
| Filtration fraction %<br>Difference of mean versus control | 33 0±5 9 | −2 5±4 5<br>p < 0 2 | −0 7±2 7<br>p < 0 4 | −? 6±3 5<br>p < 0 2 |
| Serum Na mEq/l<br>Difference of mean versus control | 145±4 3 | −0 3±4 8<br>p < 0 9 | −2 6±2 6<br>p < 0 05 | −1 50±2 81<br>p < 0 3 |
| Serum protein g/100 ml<br>Difference of mean versus control | 6 25±0 73 | −0 27±0 15<br>p < 0 001 | −0 51±0 27<br>p < 0 001 | −0 71±0 57<br>p < 0 05 |
| Blood pressure mmHg<br>Difference of mean versus control | 133±10 | 0±4<br>p < 0 9 | −3±3<br>p < 0 4 | −3±?<br>p < 0 4 |
| n | 8 | 8 | 8 | 4 |

The mean value of the right the and left kidney has been used for statistical analysis

TABLE I B General effects of infusing 150 mEq Na solution 1 2 and 3 % of the body weight

| | Control m±1 S D | 1 expansion | 2 expansion | 3 expansion |
|---|---|---|---|---|
| GFR ml/min<br>Difference of mean versus control | 27 5±13 1 | 0 1±4 8<br>p < 0 9 | −1 4±5 8<br>p < 0 5 | −3 8±5 ?<br>p < 0 ? |
| Filtration fraction<br>Difference of mean versus control | 30 5±6 3 | 0 1±6 4<br>p < 0 9 | −6 1±4 3<br>p < 0 001 | −5 6±5 7<br>p < 0 1 |
| Serum Na mEq/l<br>Difference of mean versus control | 146 ±? 7 | ? 5±2 1<br>p < 0 1 | 2 6±2 7<br>p < 0 1 | 3 6±3 8<br>p < 0 1 |
| Serum protein g/100 ml<br>Difference of mean versus control | 6 66±0 50 | −0 44±0 59<br>p < 0 2 | −1 09±0 ?8<br>p < 0 001 | 1 30±0 31<br>p < 0 001 |
| Blood pressure mmHg<br>Difference of mean versus control | 135±10 | −3±6<br>p < 0 5 | −1±6<br>p < 0 8 | 3±10<br>p < 0 7 |
| n | 5 | 5 | 5 | 5 |

The mean value f the right and the left kidney has been us d for statistical analy is

TABLE I C Gen ral effects of infusing 600 mEq Na solution 1 ? and 3 of the body weight

| | Control m±1 S D | 1 expansion | ? expansion | 3 expansion |
|---|---|---|---|---|
| GFR ml min<br>Diff rence f mean v rsus control | 26 8 4 1 | 2 5 10 1<br>p < 0 4 | 3 0 6 3<br>p < 0 1 | −4 1−6 0<br>p < 0 05 |
| F ltrati n fraction<br>D fferenc f m an ersus control | 3? 9 3 2 | 0 8−10 9<br>p 0 7 | −? 4 7 0<br>p 0 ? | −4 ? 7 9<br>p 0 1 |
| Serum Na mE l<br>Difference of mean v rsus c ntr l | 145 ? 7 | 6 4 4 1<br>p 0 01 | 11 1 4<br>p 0 001 | 17 7 8 4<br>p 0 001 |
| Serum prot g 100 ml<br>Difference of m an r us c ntrol | 6 14 0 49 | −0 ?7 0 ?<br>p < 0 001 | 0 9 0 14<br>p < 0 001 | 0 ?? 0 49<br>p 0 001 |
| Bl d pre ure m Hg<br>Diff rence f m n rsu ntr l | 135 1? | −1 2<br>p 0 2 | 4<br>p < 0 | 5 7<br>p 0 |
| n | 6 | 6 | 6 | 6 |

The mean value f th r ght n l th l ft k dney ha be n u ed f r stat t cal ar l

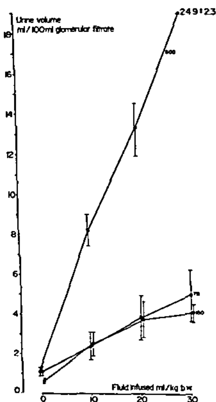



Fig 1

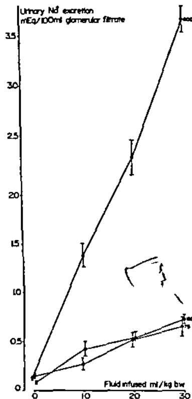



Fig 2

Fig 1 The diuretic response to the infusion of 75 150 and 600 mEq saline solution The filled circles represent the mean values the bars the standard errors of mean

Fig 2 The natriuretic response to the infusion of 75 150 and 600 mEq solutions The filled circles represent the mean values the bars the standard errors of mean

## Results

In 19 animals the effects of the 75 150 and 600 mEq NaCl solutions were examined Some general effects found in these experiments are summarized in Table I a b and c The glomerular filtration rate (GFR) did not change with the 75 mEq and 150 mEq solutions whereas the 600 mEq solution produced a slight fall in the GFR when 20 and 30 ml fluid/kg b wt had been given The filtration fraction did not change with the 75 mEq solution but fell with borderline significance when 2 and 3 % of the b wt had been given with the 150 mEq solution and when 3 % of the b wt had been given with the 600 mEq solution With the 75 mEq solution the serum Na concentration sometimes fell but the fall was generally not significant The 150 mEq solution resulted in a moderate but barely significant increase in serum Na concentration When the animals were expanded with the 600 mEq Na solution there was a continuous increase in the serum Na concentration that was

TABLE II Na reabsorbed mEq/100 ml glomerular filtrate m ± 1 S D

| | Control | Infusion of 10 ml/kg BW | Infusion of 20 ml/kg BW | Infusion of 30 ml/kg BW |
|---|---|---|---|---|
| 75 mEq solution | 14 36 ± 0 49 | 14 09 ± 0 41 | 13 76 ± 0 59 | 13 87 ± 0 58 |
| n | 8 | 8 | 8 | 4 |
| 150 mEq solution | 14 55 ± 0 29 | 14 40 ± 0 31 | 14 30 ± 0 43 | 14 18 ± 0 49 |
| n | 5 | 5 | 5 | 5 |
| 600 mEq solution | 14 38 ± 0 29 | 13 99 ± 0 65 | 13 32 ± 0 83 | 12 90 ± 0 58 |
| n | 7 | 7 | 7 | [illegible] |

The mean value of the right and the left kidney has been used for statistical analysis

TABLE III A Effects of infusing 300 mEq Na solution 1 and 3 of the body weight

| | Control ± 1 S D | 1 expansion | 3 % expansion |
|---|---|---|---|
| GFR ml/min | 15 ± 1 8 | −0 2 ± 2 8 | 0 5 ± 2 3 |
| Difference of mean versus control | | p > 0 9 | p < 0 7 |
| Filtration fraction | 34 4 ± 6 9 | −5 6 ± 6 6 | −8 4 ± 8 4 |
| Difference of mean versus control | | 0 2 > p > 0 1 | p < 0 2 |
| Serum Na mEq l | 145 8 ± 2 8 | 4 3 ± 0 5 | 9 8 ± 1 5 |
| Difference of mean versus control | | p < 0 001 | p 0 001 |
| Serum protein g 100 ml | 5 78 ± 0 37 | 0 44 ± 0 21 | −1 15 ± 0 36 |
| Difference of mean versus control | | p < 0 02 | p < 0 01 |
| Blood pressure mmHg | 131 ± 5 | 2 5 ± 5 | 0 ± 4 |
| Difference of mean versus control | | p < 0 4 | p > 0 9 |

The mean value of the right and the left kidney has been used for statistical analysis

TABLE III B Diuretic and natriuretic effects of infusing 300 mEq Na solution

| | Control | 1 % expansion | 3 % expansion |
|---|---|---|---|
| V ml 100 ml GF | 2 16 1 58 | 7 0 ± 4 0 | 32 9 ± 11 5 |
| UNa V mEq 100 ml GF | 0 31 0 30 | 1 17 0 62 | 4 3 ± 1 81 |
| n | 4 | 4 | 4 |

The mean value of the right and the left kidney has been used for statistical analysis

highly significant already after 1 % of the b wt had been given All solutions resulted in a progressive fall in serum protein concentration apparent already when 10 ml fluid/kg b wt had been given The fall was most pronounced with the 600 mEq solution The arterial blood pressure did not change regardless of whether the 75 150 or 600 mEq solution was used

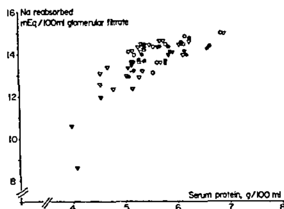



Fig 3 The relationship between serum protein concentration and Na reabsorption The filled circles represent values obtained with the 75 mEq solution the unfilled circles those obtained with the 150 mEq solution the filled triangles those obtained with the 300 mEq solution and the unfilled triangles those obtained with the 600 mEq solution

The diuretic and natriuretic responses when the constant infusion rate was given are illustrated in Fig 1 and 2 Urine volume and urinary sodium excretion increased at the same rate with the 75 and the 150 mEq Na solutions When the 600 mEq Na solution was used the diuretic and natriuretic responses were much more pronounced Since it has already been shown (Table I c) that an increase in serum Na concentration was sometimes obtained the natriuresis cannot only be attributed to adaptive changes in tubular Na transport but also in some cases to increases in the filtered load of Na While looking for the signals that could have evoked the adaptive changes it was therefore found more justified to express the effect not as excreted Na but as Na reabsorbed per 100 ml glomerular filtrate The effect of saline infusion on Na reabsorption with the 75 150 and 600 mEq solutions are summarized in Table II

Table II a and b show the effect of infusing the 300 mEq solution in 4 animals In those studies the infusion rate was adjusted so that the fluid balance should be maintained positive The infusion rate was then higher than in the experiments using 75 150 and 600 mEq Na solutions The GFR did not change The filtration fraction generally fell even though not significantly The serum Na concentration increased and the serum protein concentration fell There was a pronounced diuretic as well as natriuretic response

It is generally agreed that the peritubular oncotic pressure is an important factor in the control of tubular Na reabsorption (Spitzer and Windhager 1970 Brenner and Troy 1971 Imai and Kokko 1972) The oncotic pressure in the peritubular capillaries will be dependent on the serum protein concentration as well as on the filtration fraction The relationship between tubular Na reabsorption and serum protein concentration is illustrated in Fig 3 The values from all experiments are included

The correlation 0 68 was found to be significant ($p < 0\ 001$) Yet the relationship does not appear to be linear When the serum protein concentration falls below 5 15 g/100 ml the inhibition of tubular Na reabsorption appears to be more pronounced When the individual groups were examined statistically the correlatio

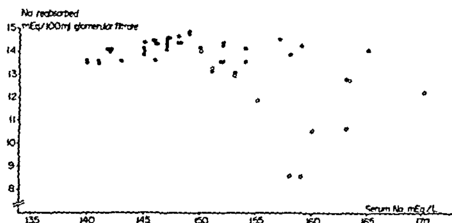



Fig 4 The relationship between serum Na concentration and Na reabsorption The filled circles represent values obtained when the serum protein concentration is above 5 15 g/100 ml The unfilled circles represent values obtained when the serum protein concentration is below 5 15 g/100 ml.

between reabsorbed Na and serum protein concentration was significant for each saline solution used The correlation coefficient (r) was 0 40 ($0\ 05 > p > 0\ 02$) for the 75 mEq solution 0 37 ($0\ 01 > p > 0\ 001$) for the 150 mEq 0 66 ($p < 0\ 001$) for the 600 mEq solution and 0 76 ($p < 0\ 001$) for the 300 mEq solution

When the infusion of 300 and 600 mEq Na solutions were given there was a progressive rise in the serum Na concentration This allowed for an analysis of the relationship between serum Na concentration and tubular Na reabsorption (Fig 4) A significant indirect correlation was found ($0\ 01 > p > 0\ 001$) When analysing this relationship it should however be remembered that the serum Na concentration generally increased parallel to but independent of the fall in serum protein concentration The obtained relationship between serum Na concentration and reabsorbed Na might therefore be false and a reflection of the relationship between serum protein concentration and reabsorbed Na To examine this the relationship between serum Na concentration and reabsorbed Na was again analyzed when the observations made at serum protein values lower than 5 15 g/100 ml (unfilled circles in Fig 4 had been excluded No relationship was then obtained ($r = 0$ When the individual groups were examined statistically great variations were found When the 150 and 600 mEq solutions were used no significant correlation was obtained When the 75 mEq solution was used there was a significant ($p < 0\ 001$) direct relationship r 0 85 between serum Na concentration and reabsorbed Na It should be noticed that the infusion of the 75 mEq solution sometimes resulted in a fall in serum Na concentration and the lowest serum Na values observed were obtained in the 75 mEq group The values observed for serum Na concentration in the 75 mEq expt ranged between 137 and 147 mEq/l Only with the 300 mEq solution a significant indirect relationship between serum Na and reabsorbed Na was obtained In this group however the highest degree of correlation between serum Na and serum protein concentration was also found

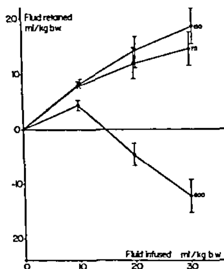



Fig 5 The relationship between fluid infused and fluid balance when the 75 150 and 600 mEq solutions are used The filled circles represent the mean values and the bars the standard errors of mean

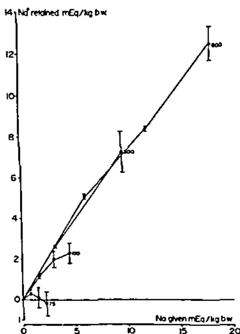



Fig 6 The relationship between Na given i v and Na balance when the 75 150 and 600 mEq solutions are used The filled circles represent the mean values and the bars the standard errors of mean

The effects of the saline infusion on fluid and Na balance are illustrated in Fig 5 and 6 The balance has been calculated as the difference between what has been infused and what has been excreted in the urine The fluid was given i v The experiments only lasted for 2—3 h and diarrhea was never encountered It would therefore seem justified to exclude fecal excretion as a factor influencing the balance Fig 5 illustrates the relationship between fluid given and fluid retained when the

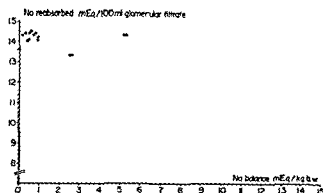



Fig 7 The relationship between Na balance and Na reabsorption The filled circles represent values obtained when the serum protein concentration is above 5 15 g/ml The unfilled circles represent values obtained when the serum protein concentration is below 5 15 g/100 ml

75 150 and 600 mEq were given (The results from studies with the 300 mEq solution are not included since the fluid balance was deliberately continued) The effect of the saline infusion on the fluid balance did not differ from when the 75 and 150 mEq solutions were used The fluid balance became increasingly positive but the relationship between retained and given fluid declined as the infusion progressed Thus 70 to 90 % of the given fluid was retained when 10 ml/kg b wt had been given while 45 to 60 % of the given fluid was retained when 30 ml/kg b wt had been given When the hypertonic saline solution was given the fluid balance became negative when more than 10 ml/kg b wt had been given

The effect of saline infusion on Na balance is illustrated in Fig 6 The Na balance has been related to the amount of Na given The effect on Na balance differed when the 75 and 150 mEq solutions were used Paradoxically the positive changes in Na balance were best corrected for when the 75 mEq solution was used When the 150 mEq solution was given the Na balance became increasingly positive but the relationship between retained Na and given Na appeared to decline as the infusion progressed When the hypertonic saline solutions were used the Na balance became positive almost at the same rate as Na was given No decline in the relationship between Na retained and Na given was observed Fig 7 demonstrates the relationship between reabsorbed Na and Na balance If the observations made when the serum protein concentration was below 5 15 g/100 ml are excluded no relationship was obtained between the two parameters A statistical analysis of the relationship between Na balance and Na reabsorption has also been made in each

TABLE IV Relationship between retained Na and H O

| Amount infused fluid ml kg b wt | 75 mEq Na amount fluid retained ml kg | Na retained/H O mEq l | 150 mEq Na amount fluid retained ml kg | Na retained mEq/l | 300 mEq Na amount fluid retained ml kg | retained Na /retained H O mEq/l | 600 mEq Na amount fluid retained ml kg | Na retained mEq l |
|---|---|---|---|---|---|---|---|---|
| 10 | 7 8 0 | [illegible] | [illegible] | 144 9 | 7 1 | 49 19 | 4 3 _ 2 0 | 1819 ± 10? |
| 20 | 11 8 7 3 | [illegible] | 14 1 | 133 18 | | | — | [illegible] |
| 30 | 9 1 ± 1 7 5 | | 18 4 | [illegible] 5 | 10 5 8 [illegible] | 137 1831 | — | [illegible] |

group In none of the groups a significant correlation was found Table IV demonstrates the relationship between retained Na and retained fluid Both with the 75 and 150 mEq solutions the relationship between retained Na and retained fluid fell far below the relationship between Na and fluid in the infused fluid Yet there was no significant fall in the serum Na concentration with the 75 mEq solution and actually a small rise in serum Na concentration with the 150 mEq solution When the hypertonic saline solution were used the relationship between Na given and fluid given by far exceeded the serum Na concentration already when 10 ml/kg b wt fo the saline solutions had been infused

## Discussion

Several observations suggest that the infused solutions are almost immediately distributed in both the intra and extracellular spaces When more than 10 ml/kg b wt of the 600 mEq solution had been given fluid balance became increasingly negative Yet serum protein concentration remained low Thus water had been rapidly withdrawn from other spaces into the vascular space When the 75 mEq solution was infused the relationship between retained Na and retained water falls far below the serum Na concentration Yet serum Na concentration is still maintained when 15 ml fluid/kg b wt has been retained This strongly suggests that the infused fluid is almost instantaneously distributed both in the extracellular and intracellular space When the hypertonic saline solutions are used the Na balance often exceeds 5 mEq/kg b wt The relatively moderate increases in serum Na concentration then obtained can only mean that the infused Na was rapidly distributed in the intracellular space as well as within the extracellular space A simple calculation shows this When 30 ml/kg b wt of the 600 mEq Na solution had been infused the Na balance had increased 12 4 mEq/kg b wt It is assumed that the extracellular fluid constitutes 200 ml/kg b wt If the infused Na would only be distributed in the extracellular space the expected increase in Na concentration would be $1000 \times 12\ 4/200 = 62$ mEq/l The observed increase in Na concentration of the plasma was however only 17 7 mEq/l It is thus obvious that saline infusion will not only result in extracellular volume expansion but might also immediately effect the total fluid and Na balance and that the latter effect will depend on the tonicity of the infused fluid

The infusion of hypo- iso- and hypertonic saline solutions consistently resulted in increased urine flow and a natriuresis The magnitude of the response v is largest with the hypertonic solutions Increased urinary Na excretion might be due either to an increase in filtered Na or to a fall in reabsorbed Na or both The glomerular filtration rate did however not increa e by the infusions the amount of filtered Na was therefore only increased when the serum Na concentration was elevated Thu increased diuresis accomplished by increa ed filtered load can be regarded as a passive over flow mechanism In contrast inhibition of tubular Na reabsorption must be considered an adaptive mechanism by which the organism tries to compensate for the changes in fluid and/or Na accomplished by the iv infusion

The amount of reabsorbed Na was significantly related to the serum Na concentration only when the 75 mEq solution was given. When the serum Na concentration rose above 147 mEq/l the amount of reabsorbed Na did not increase further but became constant. This suggests that the tubular Na reabsorption is working as a saturated system under conditions of a serum Na concentration in the upper limit of normal. This is in agreement with previous findings that hyponatremia enhances Na reabsorption (Davis *et al.* 1970) but that hypernatremia fails to do so (Puschett *et al.* 1971). It was also found that increases in serum Na concentrations do not have any inhibitory effect on the net amount of reabsorbed Na. This is somewhat in contrast to what has been reported by some authors (Blythe and Welt 1963, Kamm and Levinsky 1965). In those earlier works, however, no attempt has been made to separate the various factors that could influence urinary sodium excretion.

The Na balance did not seem to have any effect on the total amount of reabsorbed Na. This is in accordance with a recent report on the effect of chronic alterations in salt balance (Willis *et al.* 1972).

When looking for possible signals to the adaptive factor of the saline induced diuresis, the mechanisms by which tubular Na reabsorption take place has to be regarded. It is generally agreed that water is reabsorbed passively and that the driving force for this is the osmotic gradient created by the active reabsorption of Na (Giebisch 1972). When the Na ions have been pumped out of the tubular cells they will accumulate in the intercellular spaces close to the apical cellular junctions. This will result in a local hypertonicity that will attract water from the tubular lumen either *via* the tubular cells or directly through the cellular junctions. The transport of the reabsorbed fluid from the interstitium into the peritubular capillaries is dependent on hydrostatic (Martino and Earley 1967, Lewy and Windhager 1968) and oncotic (Spitzer and Windhager 1970, Brenner and Troy 1971, Imai and Kokko 1972) pressure gradients. It seems unlikely that saline infusion would diminish the hydrostatic pressure gradient so that the transport of water and Na into the peritubular capillaries would be delayed. On the other hand it is most likely that changes in oncotic pressure gradients compatible with changed fluid and Na reabsorption occurred. The serum protein concentration fell uniformly and a significant direct relationship between serum protein concentration and reabsorbed Na could be demonstrated with each saline solution used. When the observations from all studies were plotted together, the relationship between serum protein concentration and tubular Na reabsorption appeared to have a rapid and a slower phase. One reason for this might be that the oncotic pressure in the peritubular capillaries is not a direct function of the serum protein concentration in the systemic circulation. The ... tein content in the peritubular capillaries has been further ... trated by ... ering process. When the 1 0, 300 and 600 mEq Na solutions ... the ... enerally resulted in a fall in the filtration fraction. Therefore ... would ... te the effect of the low serum protein concentration on ... reab... Another possible explanation for the appearance of the

relationship between serum protein concentration and reabsorbed Na might be that the measured amount of reabsorbed Na represents the amount reabsorbed in the entire nephron It has only been proved that the oncotic pressure in the peritubular capillaries effects the fluid and Na reabsorption in the proximal tubule It might well be that inhibition of Na reabsorption in the proximal tubule is to a certain extent compensated for by increased Na reabsorption in the more distal parts of the nephron Some previous reports indicate that this is the case (Howards *et al* 1968 Higgins 1971)

The homeostatic efficiency of the diuretic response is dependent on the tonicity of the infused fluid The saline induced diuresis is mainly signalled by the serum protein concentration which in turn is determined by the fluid content in the vascular space If however the tonicity of the infused fluid deviates too much from that of blood redistribution of fluid will occur among the spaces and the fluid composition of he vascular space is no longer representative for the total fluid balance Thus when hypertonic saline is given fluid balance becomes negative but the serum protein concentration remains low and will continue to signal inhibition of tubular water and Na reabsorption Despite the exaggerated effect on fluid balance the diuresis during the infusion of the 600 mEq Na solution has very little effect on the accumulation of Na When the 75 and 150 mEq Na solutions are given fluid is accumulated first rapidly and then more slowly The animals are however somewhat dehydrated in the beginning of the study The first rapid accumulation of fluid might therefore only be a physiological correction When the animals are infused with more than 10 ml/kg b wt the rate of the accumulation is approximately half of the rate of infusion Thus the kidneys are fairly efficient in correcting the fluid balance when hypo- and isotonic solutions are given The Na balance is also fairly well controlled As has already been pointed out the infused fluid is probably immediately distributed in the intra as well as in the extracellular spaces Thus Na and fluid balance should ideally not be corrected in proportion to the Na concentration in plasma but rather in proportion to the average Na concentration of the intra *and* extracellular spaces *i e* around 55 mEq/l (Gamble 1954) Paradoxically the Na balance was best corrected for when the hypotonic saline solution was given *i e* when the need for correction was least pronounced This suggests that the effect of saline induced diuresis on Na balance will only be secondary to its effect of fluid balance

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# Glycogen Depletion Pattern in Muscle Fibres of Trotting Horses

By

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## Abstract

Lindholm A H Bjerneld and B Saltin *Glycogen depletion pattern in muscle fibres of trotting horses* Acta physiol scand 1974 *90* 475—484

Five trained standardbred horses were studied trotting at two speeds (300 and 500 m×min $^{-1}$) for 4 h and 1 h respectively and one horse at maximal speed Their mean muscle glycogen content was reduced from 103 to 43 mmol×kg $^{-1}$ in the 1 h experiment and from 119 to 48 mmol×kg $^{-1}$ in the 4 h experiment At maximal trotting speed muscle glycogen declined from 88 to 46 mmol×kg $^{-1}$ in 3 aggregate min of exercise Glycogen depletion patterns in slow twitch (ST) fast twitch high oxidative (FTH) and fast twitch (FT) fibres were estimated on the basis of the periodic acid Schiff reaction The ST and FTH fibres were depleted at all exercise intensities These fibres then probably sustained almost all the exercise at slow trotting speeds as long as their glycogen stores were not depleted After prolonged trotting and at high speeds there appeared to be a progressive increase in FT fibre recruitment Liver glycogen was reduced from 151 to 95 mmol×kg $^{-1}$ in the 1 h run and from 116 to 19 mmol×kg $^{-1}$ in the 4 h run respectively The fatigue found in horses trotting for long periods of time or at high speeds could be related to a selective depletion of glycogen in the ST and FTH fibres

Skeletal muscles of horses are rich in glycogen which is partially depleted during exercise (Lindholm and Saltin 1974) Horse skeletal muscle like most other animal muscle contains 3 major fibre types (Ashmore and Doerr 1971 Barnard *et al* 1971 Lindholm and Piehl 1974) In addition to the slow (ST) and fast twitch fibres (FT) found in man (Gollnick *et al* 1972 a) horses also have fast twitching fibres whose oxidative capacity appears to be histochemically almost as large as ST fibres

Selective glycogen depletion in the different muscle fibre types has been demonstrated in Man depending on exercise intensity and duration (Gollnick *et al* 1972 b 1973 a b) The ST fibres are the first to be depleted of glycogen during submaximal exercise and FT fibres are also depleted after 2—3 h of exercise These results suggest preferential use of ST fibres during the early phase of prolonged exercise

Selective glycogen depletion in the muscle fibres of rats or guinea pigs after running has also been observed The depletion pattern is somewhat different from that found in Man and not so certain since these animals have 3 fibre types with ST

fibres low in glycogen (Edgerton *et al* 1970, Armstrong Shepherd and Gollnick 1973) The horse also has 3 major fibre types but all are rich in glycogen In order to evaluate the use of the 3 major fibre types, the glycogen depletion pattern in the muscle fibres was studied in horses trotting at different speeds and for different periods of time Moreover, as the horse is very co-operative an attempt was made to exercise the horses to exhaustion, thereby making it possible to relate metabolic findings to fatigue

## Material and methods

Five 3—4 year old standardbred race horses (4 males and 1 female) were studied The horses were trained regularly and were in good physical condition. Two of the horses (OB and FC) were raced competitively 2—3 weeks before this study Muscle and rectal temperatures were determined by electro-thermometry (Electric Universal Thermometer type TE 3) Blood samples were taken from the jugular vein Glucose determinations were made using whole blood kept in plastic tubes containing sodium fluoride and stored in ice-cold water until analyzed by the glucose oxidase method (Hjelm and de Verdier 1963) For analyses of blood lactate 0 2 ml of whole blood were immediately pipetted into 1 0 ml of ice cold 0 6 N $HClO_4$ and lactate determined using a micro-modification of an enzymatic method (Scholz *et al* 1959) The blood sample remaining was centrifuged and the plasma free fatty acid (FFA) concentration determined (Trout Estes and Friedberg 1960)

Muscle biopsies were taken from the gluteus medius muscle of the hindquarters using the needle biopsy technique (Bergström 1962) adapted to horses (Lindholm and Piehl 1974) Trans thoracic liver biopsies were taken with a liver biopsy needle 2 mm in diameter Samples were sucked into a plastic syringe The incision was made from behind in the sixth intercostal space on a line drawn from the trochanter major of the tuber coxae to the lateral tuberosity of the humerus on the right side of the horse

The muscle sample was carefully freed from blood connective tissue and fat and divided into 2 parts After freezing in liquid nitrogen one piece was used for glycogen and triglyceride determinations and the other sample was used for histochemical analysis

Total glycogen (Karlsson Diamant and Saltin 1970) and triglyceride contents (Chernick 1969) were determined and expressed on a wet weight basis Glycogen was expressed in mmoles of glucose units In the present study the water content of the muscle increased 2 % after 4 h of trotting and 1—2 % after 30 and 60 min of fast trotting These small increases in muscle tissue water only had a slight effect on glycogen levels and did not alter the present study's basic findings

The muscle sample for histochemical analysis was mounted on a specimen holder and immediately frozen in liquid nitrogen Transverse serial sections (10 μm) were cut in a cryostat at −20 C and mounted on cover glasses for histochemical staining Muscle fibres were identified on the basis of the staining intensity for myofibrillar adenosine triphosphatase (ATPase) and reduced nicotinamid adenine dinucleotide diaphorase (NADH-diaphorase) which was estimated as described by Padykula and Herman (1955) and Novikoff Shin and Drucker (1961) respectively The myofibrillar ATPase staining is an indication of slow twitch or fast twitch fibre characteristics and the NADH-diaphorase staining is an indication of the oxidative capacity of the fibres (Barnard *et al* 1971) Muscle fibres were identified as slow twitch high oxidative (ST) fast twitch high oxidative (FTH) and fast twitch low oxidative (FT) This system of classifying muscle fibres is similar to the one suggested by Peter *et al* 1972 and the same as the one used in another study (Lindholm and Piehl 1973)

Glycogen in muscle fibres was histochemically estimated in a 16 μm thick section on the basis of the periodic acid Schiff (PAS) reaction (Pearse 1961) The intensity of PAS staining was subjectively rated as dark moderate light or negative The objectiveness of this rating has been evaluated in 2 different ways, Random selected slides have been rated by experienced individuals and they have arrived at essentially the same results The subjective rating has also been checked against a standard procedure using a photocell and a good agreement was observed (r = 0 76) (Edgerton *et al* 1973)

Trotting was performed on an 800 m track under ideal weather and track conditions 3 different trotting speeds were used 1 Slow trotting for 4 h (3 min 20 s×km⁻¹) 2 fast trotting for 1 hr (2 min×km⁻¹) and 3 maximal trotting over a 400 m distance 6 times (1 min 20 s×km⁻¹) with 5 min rest periods between heats 3 horses (MS JS CC) completed the first and second experiments whereas a fourth horse (FC) was used in the second experiment

TABLE I Individual values for glycogen in the hindquarters (gluteus medius muscle) and liver of the horse before during and after trotting at a low (left) and a fast (right) speed

| | Glycogen (glucose units) mmol × kg | | | | | | | wet weight | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Time | muscle | | | | | liver | | muscle | | | liver | |
| Horse \ h | 0 | 1 | 2 | 3 | 4 | 0 | 4 | 0 | 1/2 | 1 | 0 | 1 |
| MS | 100 | 82 | 78 | 55 | 56 | 148 | 27 | 101 | 66 | 38 | 188 | 106 |
| JS | 139 | 123 | 85 | 56 | 56 | 115 | 21 | 115 | 89 | 50 | 151 | 102 |
| GG | 119 | 86 | 69 | 58 | 32 | 85 | 10 | 127 | 78 | 64 | 236 | 171 |
| FG[4] | | | | | | | | 71 | 47 | 20 | 30 | 0 |
| m | 119 | 97 | 77 | 56 | 48 | 116 | 19 | 103 | 70 | 43 | 151 | 95 |

A fifth horse (OB) which was the best trained was used in an experiment in which maximal trotting speed was maintained for 32 s (400 m) and then repeated 6 times with 10 min rest periods between exercise sessions

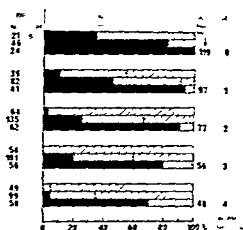
The horses were kept indoors over night before the study began and were fasted for 16 h All the horses were close to exhaustion and refused to continue trotting at the close of exercise in the first 2 studies The horse was tired in the third study but not exhausted In the 4 h of trotting exercise was interrupted after 1 2 3 and 4 h to take muscle and blood samples The horses were also encouraged to drink water during these breaks which lasted for 10—15 min but no food was given. Liver biopsies were taken at rest and at the end of exercise During the 1 h fast trotting experiment the horses rested for 5—10 min after completing 30 min of exercise blood and muscle samples were taken during these rest periods

## Results

In the 4 h expt the pace was 300 $m \times min^{-1}$ with no systematic change in speed during the exercise period Mean values for rectal and muscle temperatures at the end of this exercise period were 40 0° C and 41 9° C respectively Changes in blood lactate concentrations also reflected the work intensities of the 1 and 4 h expts Mean blood lactate at rest amounted to 0 3 (range 0 1—0 6) $mmol \times l^{-1}$ in both expts It did not exceed 1 6 $mmol \times l^{-1}$ in any horse during the 4 h expt but increased to 3 5 and 4 1 $mmol \times l^{-1}$ after 30 and 60 min of exercise respectively in the 1 h heavy-exercise expt

Muscle glycogen averaged 119 (range 100—139) $mmol \times kg^{-1}$ before the long run (Table I) Mean glycogen depletion was approximately 21 $mmol \times (kg \times h)^{-1}$ for the first 3 h but was reduced to 8 $mmol \times (kg \times h)^{-1}$ in the final hour of exercise Muscle glycogen declined from a mean value of 103 $mmol \times kg^{-1}$ at rest to 70 and 43 $mmol \times kg^{-1}$ in 30 and 60 min of exercise respectively in the 1 h exercise period Thus glycogen utilization was 3 times higher per hour in fast trotting than in slow trotting Muscle glycogen was not completely depleted in any horse at the end of any of the experiments

Based on myofibrillar ATPase and NADH-diaphorase staining 24 % of the fibres were identified as ST 50 % as FTH and 26 % as FT fibres The variation in fibre distribution among muscle samples was very small and did not deviate from mean values by more than 5 % This indicates that the fibre composition mix of horse


Fig 1

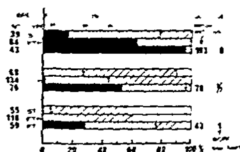

Fig 2

Fig 1 Histochemically estimated glycogen levels in slow twitch (ST) fast twitch high oxidative FTH and fast twitch, low oxidative (FT) fibres of the gluteus medius muscle of horses at rest and after 1 2 3 and 4 h of slow trotting (3 min 20 s × km⁻¹ The histogram represents the percentage of fibres with dark (black area) moderate (dense with diagonal lines) light diagonal lines or negative (white area) staining according to the PAS reaction. The numbers to the left indicate the average number of fibres used for the rating. The numbers to the right indicate the absolute concentrations of glycogen (mmoles glucose units × kg⁻¹ wet muscle and work duration h)

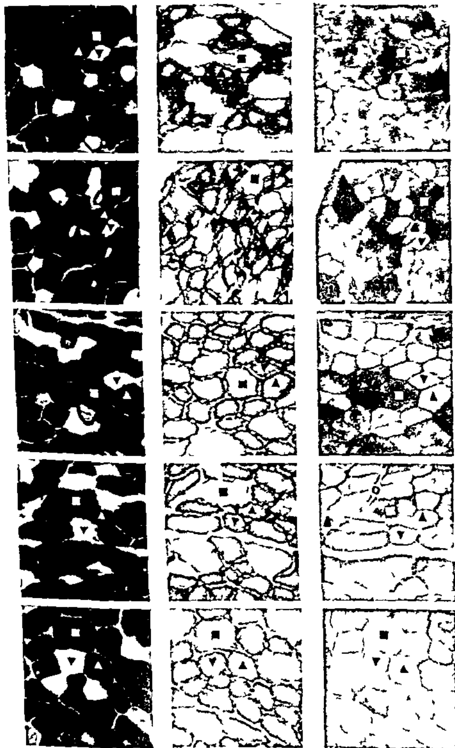
Fig 2 Histochemically estimated glycogen levels in slow twitch (ST) fast twitch high oxidative FTH and fast twitch low oxidative FT) fibres of the gluteus medius muscle of horses at rest and after 1/2 and 1 h of fast trotting (2 min × km⁻¹) For further explanation see Fig 1

gluteus medius is homogeneous with respect to fibre types there was little variation among the horses studied

At rest PAS staining was rated as dark for all FT fibres except one which was moderately stained Fig 1 78 per cent of the FTH fibres were rated as dark and the rest were moderately stained The ST fibres contained the smallest amount of glycogen But 24 % were dark based on PAS staining and 69 % were moderately stained

Both the slow and the fast trotting resulted in selective depletion of glycogen in the muscle fibres Fig 1 2) This was most apparent in the prolonged (4 h) run in which only 11 % of the ST fibres were still rated as PAS dark at the end of the first hour of work and 9 % were rated as negative 97 per cent of the FTH fibres were PAS dark and none were rated as negatively stained Almost all of the FT fibres were also rated as dark and only 6 % were moderately stained in this situation After 4 h of trotting 42 % of the ST fibres were rated as negative No ST fibres were

Fig 3 The panels show three different stains of serial sections (×170) of the gluteus medius muscle of one horse at rest after 1 2 3 and 4 h of slow trotting 3 min 20 s × km⁻¹ From left to right The left panel shows staining for myofibrillar ATPase to identify fibre types; NADH diaphorase and the right for PAS stain The three different fibres are designated as follows ST ▼ FTH ▲ and FT ■ Note that only the ST and FTH fibres are rated PAS negative after 1 and [illegible] h of exercise At the end of exercise were FT fibres were also only slightly stained The average concentrations of glycogen (from [illegible]) in this horse were 120 [illegible] and 34 mmol glucose units × kg⁻¹ wet muscle



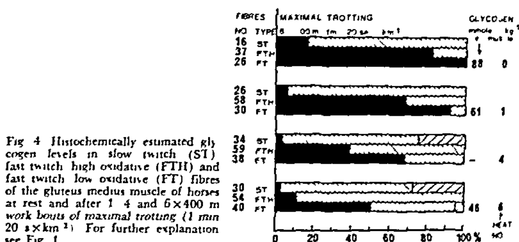



Fig. 4. Histochemically estimated glycogen levels in slow twitch (ST), fast twitch high oxidative (FTH) and fast twitch low oxidative (FT) fibres of the gluteus medius muscle of horses at rest and after 1, 4 and 6×400 m work bouts of maximal trotting (1 min 20 s×km⁻¹). For further explanation see Fig. 1.

stained dark and only 5 % were moderately stained. Some FTH fibres also became PAS negative but 5 % were still PAS dark at the end of the run. The remaining FTH fibres were moderately (30 %), lightly (47 %) or negatively (18 %) stained.

FT fibres also displayed a definite change in PAS staining but were still rated as dark after 4 h of exercise (66 %) and the remaining 34 % were rated as moderately stained. Thus glycogen depletion in the muscle fibres of the horse during the 4 h of trotting was most pronounced in ST fibres with somewhat lesser depletion in the FTH fibres. However, the staining intensity of glycogen in FT fibres decreased very slightly after 4 h of trotting. Fig. 3 shows a set of micrographs illustrating the disappearance of PAS staining in the different muscle fibres during the 4 h of trotting.

Glycogen utilization was enhanced during fast trotting and consequently the intensity of PAS staining also declined more rapidly (Fig. 4). The time sequence for glycogen depletion when comparing the different fibres was similar to the one described above. However, the PAS rating at the end of the run was the same as for ST fibres comparing the 1 and 4 h experiments. But FTH fibres and even more obviously FT fibres displayed less PAS staining at the end of fast trotting than in slow trotting. This suggests a more extensive use of FT fibres in intensive trotting. This view is also supported by results with the horse performing maximal trotting. In this intensive exercise, which only lasted for a total of 3 min, the reduction in PAS staining was as pronounced in FT fibres as in ST and FTH fibres.

The mean liver glycogen content was 116 mmol × kg⁻¹ before the 4 h expt. and this value decreased to 19 mmol × kg⁻¹ at the end of exercise (Table I). In the shorter experiment (1 h) the reduction occasioned by exercise was from 151 to 93 mmol × kg⁻¹. The blood glucose level remained fairly stable during the first 2 h of exercise in the 4 h expt. It then displayed a gradual decline in the final stages (Fig. 5). At the end of the run the mean value for blood glucose was 2.6 mmol × l⁻¹ with one horse (GG) having a blood glucose concentration of 1.5 mmol × l⁻¹. The normal resting blood glucose level for standardbred trotters is 2.8–4.4 mmol × l⁻¹ ([illegible] and Medway 196[illegible]).

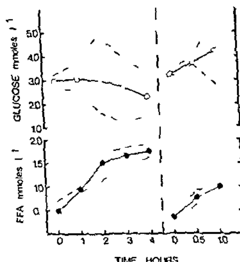



Fig 5 Mean values and the ... glucose and plasma free fatty acid ... tions in four horses ... at a ... for 4 h and at a high ...

During the 1 h of fast trotting the mean blood glucose level incr... was observed in one horse (FG) after 30 min of exercise This h... mentioned above (GG) exhibited the lowest liver glycogen values ... and end of exercise

The mean resting values for the FFA concentrations before the ... different exercise periods were 0 38 and 0 50 mmol × l⁻¹ in the ... tively FFA concentration gradually increased during exercise ... 1 75 mmol × l⁻¹ was reached in the longest run and the final val... × l⁻¹ in the shorter run The increase in FFA concentration was ... with a slight degree of variation

The extent to which muscle triglycerides were utilized during ... evaluated exactly This was partly due to the fact that a 5—10 ... in muscle triglyceride levels was observed when comparing the ... More important however was the fact that up to a threefold var... content could be observed when the determination of triglycerides was ... different parts of a muscle sample or on different samples tak... muscle on a given occasion Since the reproducibility of mea... 10 % these results indicate that lipids in horse muscles are ... as glycogen for example In two of the horses from which enough ... available muscle triglycerides were reduced in 4 h of trotting ... 43 9—21 4) to 14 1 (range 19 4—8 8) mmol × kg⁻¹ The horse ... no decrease only had a triglyceride concentration of 3 7 mmol ... exercise In the shorter experiments no definite decrease ... concentration could be noted Thus with the aforementioned ... long experiment appears to have produced a reduction in the ...

## Discussion

Skeletal muscles of horses do contain the same major fibre types as found in most other animals (Ashmore and Doerr 1971, Barnard *et al* 1971) The finding of fairly large stores of glycogen in both FT and FTH fibres and in the ST fibres of the horse is noteworthy and significant for the use of differential glycogen depletion in muscle fibres as the basis for evaluation of muscle fibre recruitment sequence during exercise The glycogen content of horse muscle was also fairly high and greater than that usually found in the muscles of man (Hultman 1967) and rats (Barnard *et al* 1971)

The fact that the ST fibres of the horse displayed somewhat greater glycogen depletion during the prolonged exercise period than FTH fibres may indicate that the latter fibre type is not as extensively involved in performing exercise as has been reported to be the case in rats (Armstrong Shepherd and Gollnick 1973) This seems to be true both at low and moderate work intensities (see Fig 1 2) The explanation for the difference between rats and horses is obscure It is true that there is a difference in the glycogen content of ST fibres but this difference should be of little importance at a low work intensity Horses are able to run at a very even pace and trotting took place on a track with no elevations Exercise intensity was therefore very even a fact which may be of some importance

FT fibres in both horses and rats were the last to be depleted and this did not occur in the low speed exercise until some of the ST and FTH fibres were depleted of glycogen The importance of exercise intensity in the recruitment of FT fibres was clearly demonstrated in the present study In trotting at a fast but submaximal pace as in the 1 h expt FT fibres were active early in the exercise This is somewhat in contrast with the circumstances found in Man FT fibres in Man are engaged in the early phase of exercise only when the work load is at a maximum and incapable of being sustained for more than 10 min in any one exercise session (Gollnick *et al* 1973 a 1974) In the maximal trotting the glycogen content of FT fibres as judged by PAS staining was strikingly reduced indicating an extensive use of these fibres when trotting is performed at this pace This is of special interest as the speed used is the one achieved by the best trotters during races The striking involvement of FT fibres at competition speeds is in accordance with the discovery of an increased cross-sectional area for these fibres at the start of training at the age of 2 years (Lindholm and Piehl 1974)

Relating the present results with the finding that the horses were fatigued in the very long run and were unable to maintain the predetermined pace in the final hour of trotting the following can be said At this point glycogen was still available to a large extent in FT and even in FTH fibres The ST fibres were by no means depleted of glycogen at this point in the exercise but the depletion pattern indicates that very many of these fibres were heavily utilized in performing the exercise It is then very likely that changes in these fibres caused by the exercise are of primary importance to the impairment of exercise capacity The data also suggest

that a determination of the total muscle glycogen content provides inadequate information on the glycogen content in working muscle fibres

A complete description of the substrate supply in trotting horses cannot be given but some important estimates can be discussed A pronounced reduction in liver glycogen was observed in the long exercise and even in the horter exercise Blood glucose levels were greatly depressed at the end of the 4 h of trotting The release of glucose from the liver cannot be calculated However, the amount of glucose available from extramuscular sources in the horse appears only to account for the smaller fraction of the total amount of carbohydrate utilized in these experiments This is due to the fact that large scale glycogen depletion was observed in the gluteus medius muscle the muscle used in the present study It has also been demonstrated that a large portion of the muscle mass of the horse exhibits depletion of a similar magnitude (Lindholm and Saltin 1974)

The increase in FFA concentration was very pronounced in both experiments and the level was far greater than other values reported for horses (Carlsson Froberg and Persson 1965) The explanation of this discrepancy is most likely to be found in the high work intensity and the long duration of experiments in the present study The study by Carlsson Froberg and Persson (1965) demonstrated that FFA was released into the blood during exercise and even taken up by the tissues illustrating the importance of extramuscular supplies of FFA during exercise even in the horse

In conclusion the present study has demonstrated that the horse can be used for metabolic studies of the effects of exercise and that horse metabolism resembles that of Man in many respects More specifically there is a preferential use of muscle fibres in the exercising horses The recruitment of muscle fibres is generally similar to the pattern described for both Man and other species In all the species studied to date ST fibres are always recruited at an early stage of exercise FTH fibres which are not found in Man are also engaged at the start of exercise by the horse regardless of work intensity Above a given trotting speed the number of FT fibres recruited is related to the exercise intensity It appears to be possible to relate the extensive use of ST fibres and possibly even FTH fibres to the signs of fatigue occurring at a late stage of prolonged exercise Moreover greatly reduced liver glycogen levels were accompanied by a drop in blood glucose levels Thus metabolic factors appear to be of importance to a horses ability to carry out endurance exercise

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From the Institute of Physiology University of Bergen Norway

# A Colloid Osmometer for Small Fluid Samples

By

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## Abstract

AUKLAND K and H M JOHNSEN *A colloid osmometer for small fluid samples* Acta physiol scand 1974 *90* 485—490

A membrane colloid osmometer for measurement on samples down to 5 μl has been developed Pellucid acryl plastic is used for most parts of the osmometer allowing visual inspection of the reference fluid chamber The osmometer base is attached directly to a Hewlett Packard pressure transducer replacing the original dome of the transducer thereby avoiding extra connections and leakage The exposed membrane area has a diameter of 3 mm and the membrane may be changed without completely disassembling the osmometer With Amicon UM 10 membranes and 0 9 % saline as reference fluid the equilibration time for measurements on human sera was 2—4 min Reduction of equilibration time to about 1 min was obtained by priming' and flushing the membrane with serum ultrafiltrate Reproducible measurements were obtained on samples down to 4—5 μl

The need for a colloid osmometer for small samples (less than 10 μl) arose in an attempt to measure colloid osmotic pressure i subcutaneous tissue fluid collected by means of implanted nylon wicks (Aukland and Fadnes 1973) Of the various types of membrane osmometers described so far the apparatus designed by Tybjærg Hansen (1961) seemed to be easiest to modify for smaller volumes than the minimum of 20 μl reported by the author During this work the whole setup was modified in several respects resulting in an osmometer which is easier to assemble than the original Tybjærg Hansen model and which permits rapid change of membrane Furthermore problems with leakage and membrane stretching have been practically eliminated and colloid osmotic pressure is measured rapidly in samples down to 4—5 μl

After development of this apparatus an osmometer with similar features has been described by Prather Brown and Zweifach (1972) However the need for greater samples (at least 50 μl) in that model and several differences in construction details seem to justify the following description of design and performance of our present colloid osmometer A preliminary report has been presented elsewhere (Aukland and Johnsen 1973)

## Construction

The construction of the osmometer is illustrated in Fig 1 and will be described in the following The clamping unit consists of 2 cylindrical blocks which will be referred to as osmometer base and top The fluid space between the semipermeable membrane surface and the transducer

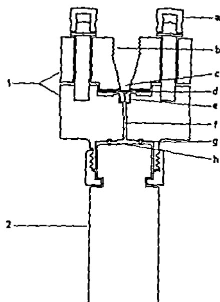


Fig 1

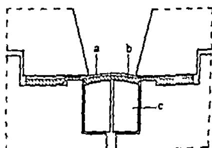


Fig 2

Fig 1 Axial section of the osmometer consisting of the osmometer base and top (1) and the pressure transducer (2) a) membrane clamping screw b) threads for connection to calibration manometer c) sample chamber d) dialyzing membrane e) membrane support f) reference fluid chamber g) O ring h) pressure sensitive membrane of the transducer

Fig 2 Detail showing the membrane in clamped position a) Semipermeable membrane layer b) porous membrane base c) membrane support

membrane will be named reference fluid chamber Base and top block of the osmometer are both made of pellucid acryl plastic allowing visual inspection of the reference fluid chamber The osmometer base is connected directly to a Hewlett Packard Type 1208 C pressure transducer thus replacing the original dome of the transducer and the rubber O ring is pressed against the transducer floor by tightening the clamping screw of the transducer From the dome over the transducer membrane a narrow channel leads up through the osmometer base to a cylindrical recess (diameter 3.5 mm) which acts as socket for the membrane support The top of the osmometer base is cup-shaped and the peripheral part of the bottom is slightly recessed leaving a 1 mm broad and 0.3 mm elevated plane ring with slightly rounded edges surrounding the membrane support socket The cylindrical membrane support also made from acryl plastic is provided with a central channel diameter 0.5 mm The diameter of the membrane support is slightly less than 3.5 mm and the bottom is furrowed allowing a thin layer of fluid between the support and its socket The top is convex and when in place its highest point levels with the plane ring surrounding the support socket

The semipermeable membrane is thereby supported in the periphery—where it is clamped down between the two osmometer blocks by tightening the clamping screws—and in the center by the top of the membrane support During clamping the plane ring surrounding the support socket is pressed into the porous base of the dialyzing membrane which inside the ring tends to bulge into the shallow groove created by the convex surface of the membrane support as shown in Fig 2 This construction causes minimal distortion of the semipermeable membrane surface and effectively prevents lateral diffusion in the membrane The sample chamber is funnel shaped and provided with threads for connection to a calibration manometer The edge of the opening facing the membrane is slightly rounded and has a diameter of 3 mm thus leaving a free [illegible] area of about 7 mm²

## Assembly and operation

Saline 0.9 % previously boiled with reflux condensation is filled into the reference fluid chamber held in upside down position and closed with a finger tip The trans-

ducer is loosely connected to the osmometer base turned to upright position and further tightened so that the central channel is completely filled Excess saline is filled into the cylindrical recess of the osmometer base and the membrane support is placed into its socket During this procedure it is important that no air bubbles are trapped in the reference fluid chamber The membrane is then mounted and clamped between the osmometer blocks by tightening the clamping screws by hand Too rapid tightening of the screws will cause a marked pressure rise in the reference fluid chamber and may separate the semipermeable membrane layer from its porous supporting base resulting in increased compliance of the reference fluid chamber The pressure is therefore observed during clamping and should not be allowed to rise above 50 mm Hg When clamped the membrane is situated in the cylindrical recess on top of the osmometer base and is still bathed in reference fluid when the osmometer top is lifted The membrane can therefore be exchanged without disconnection of the osmometer base from the transducer thus obviating refilling of the reference fluid chamber

*Membrane* — Amicon UM 10 membranes were used mainly because of their relatively high hydraulic conductivity (according to the manufacturer 0 0072 $\mu^3/\mu$ cm H O) and pore diameter which should safely exclude all serum proteins A single membrane could be used for several days without change of response characteristics

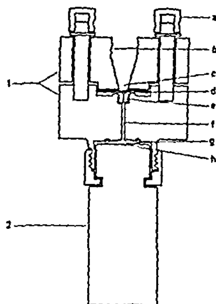
*Pressure measurement* — Pressure was recorded with a Sanborn 150 recorder using a paper speed of 0 25 mm/s Calibration was carried out by means of a simple water manometer connected to the top of the osmometer applying subatmospheric pressures of 0—30 mm Hg The pressure could be read to the nearest 0 5 mm Hg and no deviation from linearity of the transducer/recorder system was observed within this pressure range

*Baseline* — The pressure obtained by placing protein free fluid in the sample chamber gives the baseline representing zero colloid osmotic pressure Minor fluctuations of the baseline are thus easily detected and corrected

*Compliance* — The compliance of the lower part of the reference fluid chamber including the transducer membrane and the dome was tested by connecting the osmometer base to a partly water filled capillary tube of known cross section area By applying varying pressures and observing the displacement of the meniscus the compliance was found to be about 0 06 $\mu$l/100 mm Hg which is about twice the value given by the manufacturer for the transducer with its original dome

## Performance

The performance of the osmometer was tested with different human sera giving colloid osmotic pressures in the range of 24—30 mm Hg The scatter is of the same magnitude as that reported by other investigators and presumably reflects normal variation in serum protein concentrations (Ladegaard Pedersen 1967 Zweifach and Intaglietta 1971) The reference fluid chamber was filled with 0 9 % saline which in some experiments was also used for flushing the sample chamber between measure



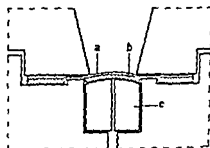




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## Assembly and operation

Saline 0 9 % previously boiled with reflux condensation is filled into the reference fluid chamber held in upside down position and closed with a finger up The trans

TABLE 1 Repeated measurements on identical serum samples Collo d osmotic pressure in mm Hg

| Day No | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Sample No | | | | | | |
| 1 | 25 0 | 25 0 | 25 0 | 25 5 | 25 0 | 26 0 |
| 2 | 25 5 | 25 5 | 25 0 | 25 5 | 26 0 | 25 0 |
| 3 | 26 0 | 25 0 | 25 0 | 25 0 | 25 5 | 26 0 |
| 4 | 25 5 | 25 0 | 26 0 | 25 0 | 25 0 | 25 5 |
| 5 | 25 5 | 25 0 | 25 5 | 25 0 | 25 0 | 25 0 |
| Mean | 25 4 | 25 2 | 25 3 | 25 2 | 25 3 | 25 5 |

*Serum dilutions* — Human sera were diluted 1/5 2/5 3/5, and 4/5 in 0 9% saline A plot of colloid osmotic pressure against relative serum protein concentrations showed the characteristic upward concave curve in agreement with that observed by other investigators (*e g* Tybjærg Hansen 1961 Intaglietta and Zweifach 1971)

*Sample si e* — By applying successively smaller samples no significant pressure deviations were observed until sample volume was reduced to less than 4—5 μl Smaller samples did not always cover the membrane surface and gave too low pressures

## Discussion

The present osmometer has considerably reduced the volume needed for measuring colloid osmotic pressure making it suitable for the present purpose of measurements on wick fluid It should also prove useful for measurements on body fluids from small animals The simple assembly procedure without previous boiling of the whole unit as necessary with several previously described models (Tybjærg Hansen 1961 Intaglietta and Zweifach 1971) makes the apparatus ready for operation in short time and if needed the membrane may be changed without completely disassembling the apparatus Possible air bubbles trapped in the reference fluid chamber are easily detected because of the transparency of all materials

The duration of the initial transient response due to equilibration of permeating molecules will partly depend on the reference fluid volume participating in this exchange For this reason the central channel of the membrane support and the peripheral connection to the transduced have been made as narrow as just to provide sufficient hydraulic conductivity thus practically reducing the exchange reference fluid volume to that contained in the porous membrane base Because of this construction the exchange reference fluid volume is rapidly equilibrated with serum as far as small molecules are concerned by priming with serum ultrafiltrate Thereby the initial transient is almost eliminated the time needed for each sample is greatly reduced and changes in sample concentrations of permeating molecules are prevented

As pointed out by Intaglietta and Zweifach (1971) lateral water transport through hydrated polymer membranes may give rise to a slight pressure decay The observation that pressure was maintained unchanged for hours shows that the membrane sealing used in our model effectively prevents lateral water transport It

also eliminates the possibility of increased compliance due to the yielding property of a rubber O ring which has been used in previous models as membrane sealing (Tybjærg Hansen 1961 Prather Gaar and Guyton 1968)

The compliance of the transducer/reference fluid chamber system was approximately 0 06 µl/100 mm Hg If the semipermeable membrane were absolutely unyielding this would also be the compliance of the whole reference fluid space and a serum sample with a colloid osmotic pressure of 30 mm Hg would cause a volume displacement of only 0 02 µl thus giving negligible dilution of a 5 µl sample The total system compliance must be somewhat greater mainly because of the elastic property of the semipermeable membrane but is not readily measured However the fact that the same colloid osmotic pressure was observed in samples of 4 to 100 µl showed that for practical purposes the dilution effect is negligible

The small day to-day variations of pressure measured on identical samples may be due to differences in room temperature and even better reproducibility would be expected if the osmometer were thermostatically controlled However a measuring error of ±0 5 mm Hg will be acceptable for most purposes

The need for a standard solution of known colloid osmotic pressure for calibration has been emphasized by Intaglietta and Zweifach (1971) who also pointed out the difficulties in providing such a solution It seems to us however that if the apparatus can be directly calibrated by a water manometer and gives a pressure response which is maintained unchanged for more than 1 h as is the case with the present model there is really no need for any further calibration By definition the stable pressure observed after applying the sample equivalates the osmotic pressure of colloids not permeating the dialyzing membrane In conclusion the present colloid osmometer allows measurements on samples down to 5 µl without loss of measuring precision compared to previously described small sample models (Tybjærg Hansen 1961 Prather Gaar and Guyton 1968) The osmometer can be made from cheap materials in a reasonably well equipped workshop It is easy to operate and permits at least 20 measurements per hour

We are greatly indebted to laboratory engineer E G Halleland whose good ideas and excellent handicraft were of decisive importance in realizing this project

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# Acceleration Stress and Effects of Propranolol on Cardiovascular Responses

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## Abstract

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Heart rate arterial pressure and cardiac output were recorded in six physically well trained young male volunteers in the sitting position at normal gravity and at 3 G acting in the head seat direction before and after beta adrenergic blockade by propranolol Studies were performed both at rest and during leg exercise at a load corresponding to 50 % of the maximal oxygen uptake After propranolol the heart rate response to increased G at rest averaged 38 % of that observed without blockade indicating that G induced cardioaccelera tion is predominantly due to sympathetic stimulation G induced decline of cardiac output was more marked after propranolol this effect being entirely due to the reduced response of the heart rate In spite of the cardioinhibitory effects of propranolol subjective G tolerance was well preserved the response of the arterial mean pressure to increased G was unaffected due to a 65 % rise in systemic vascular resistance as against 43 % before propranolol Hence sympathetic chronotropic stimulation of the heart is not essential for the circulatory defense against increased gravitational stress Transition from rest to exercise at 3 G produced a larger increase in stroke volume than in heart rate after beta adrenergic blockade the dominance of the stroke volume increase was exaggerated

Gravitational pull in the head foot direction whether produced by assumption of the erect posture or amplified by exposure to centrifugal force leads to consequences which are due largely to the effects of increased hydrostatic pressures in the vascular tree Among the immediate effects are a lowering of the mean arterial pressure at head level and a downward displacement of blood volume from the intrathoracic space (for review see Gauer and Zuidema 1961) Two of the major compensatory responses to these disturbances are tachycardia and vasoconstriction evoked through reflexes originating in arterial baroreceptors and probably in part also in baroreceptors located in intrathoracic low pressure areas The role played by reflex vasoconstriction is illustrated by the continuing fall of arterial pressure that occurs in subjects exposed to gravitational stress following autonomic ganglionic blockad

Table I. Individual dimensional and functional data

| Subj | Age years | Weight kg | Height cm | B.S.A. $m^2$ | $\dot{V}_{O_2}$ max ml/min × kg |
|---|---|---|---|---|---|
| M.R. | 21 | 80 | 190 | 2.07 | 53 |
| B.G. | 24 | 74 | 179 | 1.92 | 54 |
| S.B. | [illegible] | 70 | 176 | 1.85 | 54 |
| J.P. | 24 | 63 | 173 | 1.75 | [illegible] |
| R.H. | 21 | 78 | 178 | 1.96 | 50 |
| M.S. | 20 | 73 | 185 | 1.93 | 48 |

B.S.A. = body surface area from nomogram of Dubois and Dubois (1916)
$\dot{V}_{O_2}$-max = maximal oxygen uptake

(Brown, Wood and Lambert 1949). It remains unclear, however, whether the increase in heart rate, which is one of the most consistent effects of gravitational stress, is essential to the adequacy of the cardiovascular response. In view of the increased excretion of catecholamines in various forms of gravitational stress (Euler, Luft and Sundin 1955, Sundin 1958, Goodall 1962), it can be assumed that the cardioacceleration is at least in part mediated by sympathetic arousal. A study of the response to an agent which selectively inhibits adrenergic effects on the heart might therefore serve to elucidate the importance of cardioacceleration in the defense of circulatory homeostasis during gravitational stress. Accordingly, we have compared in human subjects the cardiovascular responses to a three fold increase of the force of normal gravity with and without the administration of propranolol, an agent which effectively inhibits adrenergic effects on the heart while exerting a relatively small influence on the response of the peripheral circulation to sympathetic stimulation (for review, see Dollery, Paterson and Conolly 1969). In addition, this study was designed to investigate the influence of propranolol on the cardiovascular responses to moderate leg exercise when performed during increased gravitational stress.

## Material and Methods

The experiments were carried out in the human centrifuge (radius = 7.4 m) at the Karolinska institutet. Six healthy, physically well-trained, male volunteers, aged 21 to 3_ years, were studied. The subjects were thoroughly acquainted with the experimental procedure before their informed consent was obtained. Individual dimensional and functional data are given in Table I. During the course of 2—4 weeks preceding the day of the experiment at least 3 preliminary sessions were conducted on separate days with each subject. These sessions, during which the subject was exposed to G profiles similar to those used in the actual experiment, with and without leg exercise, served as training periods to accustom the subject to the subjective sensations of sustained accelerative stress and to minimize any emotional impact from such stress. Resting heart rates recorded immediately prior to the experimental centrifuge runs indicated absence of any significant apprehension or excitement.

On the day of the experiment subjects reported to the laboratory within 2 h after breakfast. The intravascular catheters used for the procedure were introduced while the subject rested supine prior to his entering the centrifuge gondola. Both radial arteries were entered percutaneously at the wrist by small Teflon catheters (length = 7 cm, O.D. = 1.15 mm, I.D. = 0.75 mm). A venous catheter (length = 30 cm, O.D. = 1.7 mm, I.D. = 1.2 mm)

was introduced percutaneously into an antecubital vein and advanced so that its tip was located in the axillary vein. The subject then walked to the centrifuge room and entered the gondola, where the right radial catheter was connected to one of the two inputs of a differential strain gauge manometer (Statham P 23H) mounted in the gondola well below the level of the heart and positioned so that the sensing element was not influenced by the major G vector. To obtain the arterial pressure at heart level the other input of the strain gauge was hydraulically connected to a plastic tube which was taped onto the skin along the sternum and filled with saline up to the level of the insertion of the 4th rib. This technique afforded automatic compensation for the hydrostatic distance between the heart and the fixed strain gauge and thus permitted accurate recording of the mean arterial pressure and its changes at heart level irrespective of changes in the G level (*cf* Linnarsson and Rosenhamer 1968). Cardiac output was determined by the indicator dilution technique using indocyanine green (Cardio-Green®) and a Waters X 100 dichromatic densitometer modified electrically to minimize drift and other disturbances in the centrifuge environment. To produce dye-dilution curves with the subject in the spinning centrifuge dye injections as well as the subsequent withdrawal and reinfusion of arterial blood through the densitometer cuvette were made by a remote-controlled injection system described elsewhere (Rosenhamer 1967, 1968 b). The amount of dye injected for each determination was 1.00 ml of a 0.5 % aqueous solution of indocyanine dye and the rate of withdrawal of blood through the cuvette was 20 ml/min. To prevent clotting of blood in the cuvette withdrawal/reinfusion syringe system the syringe was siliconized and the subject given 10 000 I.U. Heparin (Vitrum) intravenously. Heart rate was obtained from chest electrodes and recorded by means of a linear beat-to-beat cardiotachometer (Lindborg, Wigertz and Ödman 1969). All signals were displayed on a strip-chart recorder (Brush Mk 200) and recorded on magnetic tape (Ampex FR 100C) for storage and subsequent detailed analysis of the variables under study. In addition the dye-dilution curves were inscribed on an XY recorder (Hewlett Packard 700 AM) at a paper speed of 4 mm/sec.

*Design of experiments:* All experiments were conducted with the subjects seated in the centrifuge gondola. The gondola accommodated an electrically braked cycle ergometer with the crank axis at the level of the gondola seat. The basic experimental plan was to compare cardiovascular responses to a change from normal to high G before and after beta adrenergic blockade and to extend this comparison to the exercising condition. For this purpose each subject participated in two experimental sessions. Each session consisted of four conditions: 1) 5 min rest at normal gravity, 2) 6 min leg exercise at normal gravity followed by a recovery period, 3) 5 min rest at 3 G and 4) 6 min leg exercise at 3 G. The subject kept his feet on the pedals also in the resting conditions. The time required to attain 3 G after starting the *centrifuge* was 9 s. The two sessions were separated by an interval not longer than 20 min during which propranolol (Inderal®) was slowly given via the venous catheter in a dose of 0.25 mg/kg b.wt. In the exercise conditions the work load was preset individually to correspond to approximately 50 % of the subject's aerobic working capacity, the pedalling rate being maintained 60 rpm. The external work provided by the cycle *ergometer was* essentially unaffected by the increased accelerative force (Rosenhamer 1968 a) and the effect of increased G on the average work performed by the leg muscles was negligible (Bjurstedt, Rosenhamer and Wigertz 1968). *Three* consecutive indicator-dilution curves were recorded in each one of the experimental conditions. The blood withdrawn during the dilution curve was reinfused into the radial artery upon completion of each dye curve. The curves obtained from the first injection in any one condition after 2 min in the resting condition and after 2.5 min in the exercise condition were regarded as "wash-out curves" and were discarded and the *cardiac output* was estimated from the mean values of the two following curves. Heart rate and arterial pressure were continuously recorded throughout the experimental sessions.

The statistical significance of differences between mean values were evaluated by applying the t test to the intra individual differences (*cf* Fisher 1948).

## Results

With exposure to 3 G in the resting condition the majority of the subjects reported a transient impairment of vision ("greyout") during the initial 15 sec of the run. After propranolol the 3 G runs at rest were subjectively experienced as less unpleasant and in none of the subjects was the initial greyout more apparent than

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(*Brown, Wood and Lambert 1949*). It remains unclear, however, whether the increase in heart rate, which is one of the most consistent effects of gravitational stress, is essential to the adequacy of the cardiovascular response. In view of the increased excretion of catecholamines in various forms of gravitational stress (Euler, Luft and Sundin 1955, Sundin 1958, Goodall 1962), it can be assumed that the cardioacceleration is at least in part mediated by sympathetic arousal. A study of the response to an agent which selectively inhibits adrenergic effects on the heart might therefore serve to elucidate the importance of cardioacceleration in the defense of circulatory homeostasis during gravitational stress. Accordingly, we have compared in human subjects the cardiovascular responses to a three fold increase of the force of normal gravity with and without the administration of propranolol, an agent which effectively inhibits adrenergic effects on the heart while exerting a relatively small influence on the response of the peripheral circulation to sympathetic stimulation (for review, see Dollery, Paterson and Conolly 1969). In addition, this study was designed to investigate the influence of propranolol on the cardiovascular responses to moderate leg exercise when performed during increased gravitational stress.

## Material and Methods

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On the day of the experiment subjects reported to the laboratory within [illegible] h after breakfast. The intravascular catheters used for the procedure were introduced while the subject rested supine prior to his entering the centrifuge gondola. Both radial arteries were entered percutaneously at the wrist by small Teflon catheters (length = 7 cm, O.D. = 1.15 mm, I.D. = 0.75 mm). A venous catheter (length = 30 cm, O.D. = 1.7 mm, I.D. = 1.2 mm)

ABLE IV Responses to change from rest to exercise at 3 G (work load corresponding to 50 of individual aerobic working capacity) Abbreviations as in Table II

| | MAP mm Hg | | HR beats/min | | CO l/min | |
|---|---|---|---|---|---|---|
| | B | A | B | A | B | A |
| est | 126±3 | 125±4 | 116±7 | 86±4 | 6 3±0 4 | 4 2±0 2 |
| xercise | 139±3 | 130±4 | 154±3 | 113±2 | 15 8±1 6 | 14 1±1 2 |
| xerc rest | 13±3 | 5±1 | 38±6 | 27±3 | 9 5±1 4 | 9 9±1 0 |
| P | < 0 01 | < 0 05 | < 0 01 | < 0 001 | < 0 001 | < 0 001 |
| | **TPR Units** | | **SV ml** | | **LVW watt** | |
| | B | A | B | A | B | A |
| rest | 1 23±0 10 | 1 85±0 17 | 57±7 | 49±4 | 1 75±0 12 | 1 15±0 06 |
| exercise | 0 56±0 07 | 0 58±0 06 | 103±12 | 126±11 | 4 81±0 43 | 4 03±0 26 |
| exerc rest | −0 67±0 07 | −1 27±0 13 | 46±8 | 77±10 | 3 06±0 37 | 2 88±0.26 |
| P | < 0 001 | < 0 001 | < 0 01 | < 0 001 | < 0 001 | < 0 001 |

*before* propranolol No visual symptoms were reported when exposure to 3 G was combined with exercise neither before nor after propranolol However exercise at 3 G was uniformly reported as more fatiguing after propranolol than before

*Responses to 3 G in the resting condition (Table II)*

*Before propranolol* Exposure to 3 G produced an 81 % increase in heart rate and a 50 % decrease in stroke volume The cardiac output showed a tendency to fall but the change was not statistically significant Arterial mean pressure at heart level showed a 27 % increase ($p < 0\,001$) the systemic vascular resistance being increased by 43 % ($p < 0\,01$) Left ventricular work was not significantly affected by the change in G level

*After propranolol* At normal gravity in the resting condition propranolol did not alter heart rate significantly but reduced cardiac output and stroke volume 27 % ($p < 0\,05$) and 31 % ($p < 0\,05$) respectively Arterial mean pressure at heart level was not significantly changed and systemic vascular resistance therefore rose 30 % ($p < 0\,05$) Left ventricular work was reduced 31 % ($p < 0\,05$)

With the change from normal gravity to 3 G heart rate increased significantly less ($p < 0\,001$) than before propranolol Cardiac output and stroke volume decreased 19 % ($p < 0\,01$) and 39 % ($p < 0\,01$) respectively Due to a substantial rise in vascular resistance (65 %) arterial mean pressure at heart level rose to about the same level as before propranolol The increase in systemic vascular resistance was significantly ($p < 0\,01$) greater than before propranolol As before propranolol left ventricular work was not significantly influenced by the change in G level

*Responses to exercise at 1 G and 3 G (Tables III and IV)*

*Before propranolol.* With a change from rest to exercise heart rate increased less at 3 G than at normal gravity (33 and 103 %, respectively, $p < 0.01$). Cardiac output showed similar increases at the two G levels and stroke volume therefore increased more at 3 G than it did at normal gravity where no significant alteration occurred. Exercise induced increases in arterial mean pressure and left ventricular work were similar at the two G levels. Reductions in systemic vascular resistance due to exercise were 51 % at normal gravity and 54 % at 3 G.

*After propranolol.* In the exercise condition at normal gravity, propranolol reduced cardiac output 20 % ($p < 0.05$). Stroke volume was not changed significantly and the decrease in cardiac output was therefore predominantly due to a reduction of the heart rate (15 %, $p < 0.001$). Left ventricular work decreased 27 % ($p < 0.01$) which was due to the diminished cardiac output as well as a 9 % decrease ($p < 0.01$) in arterial mean pressure.

Exercise induced changes in hemodynamic variables were modified as follows. At normal gravity a smaller elevation of the heart rate (68 % as against 103 % before propranolol) was compensated by a 51 % increase ($p < 0.05$) in stroke volume, this variable remaining largely unchanged before propranolol. Changes in arterial mean pressure, left ventricular work and systemic vascular resistance were similar before and after propranolol.

At the 3 G level the exercise induced elevation of cardiac output was accompanied by a larger increase in stroke volume (157 %) than before propranolol (81 %), the absolute level of this variable during exercise being increased by this drug (22 %, $p < 0.01$). *Arterial pressure increased only 4 % as against 10 % before propranolol,* which was predominantly due to the fact that the exercise induced fall in vascular resistance at 3 G was greater after than before propranolol (69 and 54 %, respectively).

## Discussion

In the present study cardiovascular homeostasis was challenged by exposure to a force environment which produced a three fold increase in the hydrostatic pressure gradient. During the course of the 3 G runs at rest, the arterial mean pressure at heart level, which averaged 99 mm Hg at normal gravity, dropped somewhat initially and subsequently rose to a final value of 126 mm Hg. Assuming an average vertical heart-to-eye distance of 30 cm, the mean perfusion pressure at eye level at 3 G can be calculated to have been on an average 66 mm Hg lower than the arterial mean pressure recorded at heart level. Thus the perfusion pressure at eye level was well above the intraocular pressure except initially, when temporary visual impairment was experienced by the majority of the subjects. The finding that propranolol did not reduce subjective G tolerance is consistent with the observations of Cohen and Brown (1969), who however used a considerably smaller dose (5 mg) in their experiments. That visual symptoms did not occur when exposure to

G was combined with leg exercise is in agreement with earlier observations by Rosenhamer (1967), and is a reflection of he exercise induced increase in arterial mean pressure that has previously been reported to occur at the 3 G level (Linnarsson and Rosenhamer 1968)

*Effects of beta adrenergic blockade at rest and in exercise at normal gravity* Propranolol reduced cardiac output at rest as well as during exercise which is in agreement with other observations (Epstein *et al* 1965 Åström 1968 Wolfson and Gorlin 1969) However the underlying mechanism differed in the two conditions The subjects were physically well trained and the average resting heart rate before propranolol was therefore low Propranolol produced no further reduction of the heart rate at rest and the decrease in cardiac output in this condition was therefore caused by a decrease in stroke volume only probably due to diminished inotropic drive During exercise on the other hand stroke volume was little affected and the propranolol induced reduction of cardiac output was primarily caused by a decrease in the heart rate which was similar to the changes reported by Epstein *et al* (1965) and Astrom (1968) These findings indicate that sympathetic stimulation of the heart contributed to maintaining the cardiac output both at rest and during exercise and that such stimulation was exerted through positive inotropic influence on the heart at rest and predominantly by a positive chronotropic effect in exercise

The reductions of left ventricular work observed after propranolol at rest as well as during exercise were similar to those reported by others (Epstein *et al* 1965 Åstrom 1968) Since the arterial mean pressure was essentially unchanged at rest which is in agreement with the observations of Robinson *et al* (1965) and Åstrom (1968) left ventricular work decreased in proportion to the decrease in stroke volume

*Sympathetic cardiac drive in the resting condition at 3 G* With the change from normal gravity to 3 G before propranolol heart rate increased 81 % the magnitude of this change being consistent with what could be expected from the results of numerous earlier investigations employing a wide range of G levels (for review see Howard 1965) After propranolol the response of the heart rate was only 38 % of that observed without this drug Thus the cardiac acceleration induced by increased gravitational stress is predominantly due to sympathetic stimulation That the increase in heart rate was not abolished by propranolol may be the result of incomplete blockade of sympathetic influence or of the operation of additional mechanisms Although it has been reported by Epstein *et al* (1965) that propranolol in a dose of 0 15 mg/kg b wt reduces the effectiveness of infused isoproterenol by at least 90 % gravitational stress may increase the sympathetic background activity to an extent that even the higher dose of 0 25 mg/kg as employed in the present experiment permits appreciable cardioacceleration of sympathetic origin However Robinson *et al* (1966) have demonstrated that the increase in heart rate induced by head up tilt is almost abolished after combined autonomic blockade produced by atropinization and propranolol in a dose of 0 25 mg/kg Furthermore recent experiments by our group (unpublished observations) have indicated that such

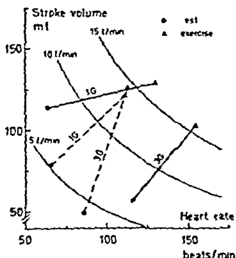



Fig. 1. Changes in heart rate and stroke volume with transition from rest to exercise at 1 G and 3 G. Solid and broken lines refer to events before and after propranolol, respectively. Curved lines indicate different levels of cardiac output.

combined blockade almost completely abolishes the heart rate response to G levels even higher than that employed in the present study. It is therefore likely that the residual heart rate response to the change from 1 G to 3 G after propranolol can in the main be attributed to baroreflex influence mediated through a reduction in vagal tone.

Before propranolol, the change from normal gravity to 3 G produced a 30 % decrease in stroke volume, which is in agreement with the 37 and 51 % reductions previously reported for the 3 G level by Lindberg *et al.* (1961) and Rosenhamer (1967), respectively, and which can be ascribed to defective cardiac filling due to G-induced downward displacement of blood volume from the intrathoracic space. Cardiac output showed a tendency to fall, but the change in this variable was less consistent than the 18 and 24 % reductions observed by the above-mentioned authors. The 27 % elevation in arterial mean pressure at heart level is similar to the changes reported by Lindberg *et al.* (1961) and Linnarsson and Rosenhamer (1968) at the same G level. It appears that simultaneous recordings of intraarterial pressure and cardiac output in man during increased gravitational stress have previously only been made by Lindberg *et al.* (1961). These authors reported a 41 % increase in systemic vascular resistance at 3 G, which is similar to the 43 % increase obtained in the present investigation.

The impaired response of the heart rate with exposure to 3 G after propranolol was accompanied by a more clearcut curtailment of cardiac output than occurred before the drug. At the same time stroke volume decreased less, and the greater G-induced decline of the cardiac output that was seen after propranolol can therefore be accounted for entirely by the weaker response of the heart rate. This indicates that during increased gravitational stress, at least within the range studied in the present experiments, sympathetic chronotropic influence on the heart contributes to some extent to maintaining the cardiac output.

It was of interest to note that with the change from normal gravity to 3 G the arterial mean pressure at heart level rose to about the same level before and after propanolol This explains the fact that the subjective G tolerance was remarkably well preserved after the drug It also means that the loss of sympathetic chronotropic drive to the heart after propranolol leading to a greater G induced decline of the cardiac output was compensated for by a more potent vasoconstrictor response The fact that the G induced vasoconstrictor response was so precisely adjusted to compensate for the impaired response of the heart rate indicates not only that the central regulation of the arterial pressure was intact after propranolol it also suggests that sympathetic chronotropic stimulation of the heart is not an essential mechanism in the circulatory defense against increased gravitational stress

*Cardiac adjustments to exercise at 3 G* The observation that a change from rest to moderate exercise without propranolol produced a much larger increase in stroke volume at 3 G than at normal gravity is in accordance with earlier observations by Rosenhamer (1967) Cardiac adjustment to exercise at 3 G therefore differed from that at normal gravity where the elevation of cardiac output was due mainly to an increase in heart rate (*cf* Fig 1) The different responses with regard to stroke volume and heart rate at the two G levels can best be explained by the fact that stroke volume was greatly lowered in the resting condition at 3 G due to downward displacement of blood volume from the intrathoracic space and by the assumption that the action of the leg muscle pump therefore permitted a relatively larger increase in stroke volume in this condition than it did at normal gravity A similar discrepancy although less conspicuous may be observed with posture shifts at normal gravity where leg exercise produces a greater increase in stroke volume in the upright than in the supine position (Bevegard Holmgren and Jonsson 1960 Wang Marshall and Shepherd 1960)

When the venous return was boosted by the action of the leg muscle pump stroke volume increased more after propranolol at normal gravity than it did without this drug propranolol also exaggerated the dominance of the stroke volume increase at 3 G (*cf* Fig 1) Both effects may be envisaged as resulting from the withdrawal of sympathetic chronotropic drive permitting an increased cardiac filling through a lengthening of the diastolic filling time and a resultant augmentation of the stroke output through the operation of the Frank Starling mechanism This mechanism may be responsible also for the fact that the absolute value of the stroke volume during exercise at 3 G was in fact greater after than before propranolol (*cf* Fig 1) This interpretation implies that in the face of a boosted venous return during exercise cardiac filling was normally to some extent limited by sympathetic acceleration of the heart rate and that this effect was especially apparent at the 3 G level

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Acta physiol scand 1974 90 501—504

From Kristinebergs Zoologiska Station Fiskebackskil Suede et Lab de Zoophysiologie
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# Des Mecanismes Biochimiques de l'Automatisme Cardiaque
## Examen de la Myxine

par

B Rybak et M Simon

Reçu le 6 Aout 1973

### Abstract

Rybak B and M Simon *On the biochemical mechanisms of the heart automaticity Study on Myxine* Acta physiol scand 1974 *90* 501—504

Kinetic titrations of cAMP in the branchial heart of *Myxine glutinosa* seem to show that cAMP is involved in automatic processes and that there are quantitative differences in cAMP and specific phosphodiesterase inside the auricle and inside the ventricle in such a way that compiling the present results with other data one explains how the auricle is the dominant pace maker in this heart.

L un de nous a montre (Rybak 1973) que l adenosine 3 5 monophosphate (cAMP) est implique dans l induction de l automatisme cardiaque en explicitant le pouvoir automatogene de $Ba^{++}$ sur pointes ventricularires isolees du cœur de *Rana esculenta* phenomene mis en evidence par Abderhalden et Gellhorn (1920 *cf* aussi le travail de precurseur de P Mathieu 1914) Les valeurs de cAMP auxquelles on arrive avec BaCl /1200 en solution de Ringer non glucosee et non phosphatee sont assez disperses mais significatives (cette dispersion peut etre la consequence d une teneur differente en ATP des pointes ventriculaires) On trouve ainsi de 1 41 a 20 nmol cAMP/mg essore de pointes inertes et de 26,30 a 52 52 nmol cAMP/mg essore de pointes en contractions regulieres ($\theta \sim 20°$ C) Le temps de latence d induction des revolutions myocardiques va de 7 a 23 min cette periode de latence peut etre raccourcie si on traite prealablement les pointes ventriculaires par un bain dans une solution hypercalcique de Ringer — 2 16 mM CaCl soit le double de la concentration normale — puis en les plongeant dans une solution de Ringer normale additionnee de $BaCl_2$/1200 le temps de latence est ramene a 1 5—2 min ceci peut s expliquer en considerant les relations de polarisation electrique membranaire developpees par $Ca^{++}$ d une part et $Ba^{++}$ de l autre (*cf* par exemple Lehmkuhl et Sperelakis 1967)

Nous avons poursuivi ici l examen de ce fait nouveau qu est l intervention du cAMP dans l automaticite cardiaque en travaillant avec le cœur branchial aneural de *Myxine glutinosa* (Greene 1902 Carlson 1904 Fange et Östlund 1954 Bloom *et al* 1961 Rybak 1960 Hoffmeister *et al* 1961 Rybak *et al* 1962)

## Techniques

Le dosage du cAMP se fait par la technique enzymatique de Butcher et Sutherland (1962) à $\theta \sim 20$ °C (Produits Boehringer Mannheim).

Le cœur branchial est isolé rapidement ouvert sous tension du minutier (Rybak 1959) pour en faciliter le nettoyage cavitaire, essoré sur papier Joseph puis broyé avec de l'eau de mer diluée selon Young (193?) à pH 7 65 et ~20 °C au Potter entraîné par un moteur. L'homogénat est centrifugé à 0 °C et 8000 × g pendant 10 min dans une centrifugeuse « International ». Nous avons standardisé la durée de préparation à 40 min, tous les temps opératoires étant chronométrés. La cinétique constituant le dosage porte sur le surnageant limpide et se pratique avec le spectrophotomètre « Beckman DU » à 265 nm (largeur de fente 0 04 mm) dans une cuvette de quartz de 1 5 ml de capacité. Les microvolumes nécessaires ont été prélevés et transférés avec des *micro-pipettes* automatiques « Gilson » et des *micro-pipettes* « Pedersen » à zéro automatique.

## Résultats

Les cinétiques de vieillissement des broyats tissulaires conservés à 0 °C donnent par extrapolation à zéro pour chaque cinétique ($t_0$ d'extraction) le taux initial de cAMP et le taux de cAMP détruit lors de la conservation. Les teneurs de cAMP initial obtenues avec environ 150 dosages sont les suivantes :

| | |
|---|---|
| cœur entier | 8,55 nmol cAMP/mg essoré $\sigma = 0,20$ |
| oreillette seule | 15 99 nmol cAMP/mg essoré $\sigma = 0,50$ |
| ventricule seul | 9 05 nmol cAMP/mg essoré $\sigma = 0,21$ |
| surnageant d'homogénat auriculaire + surnageant d'homogénat ventriculaire dans le rapport 1 : 1 | 8 4 nmol cAMP/mg essoré $\sigma = 0,2$ |

Il découle de la lecture de ces valeurs que :

1°) l'oreillette est plus riche en cAMP que le ventricule ;

2°) la concentration en cAMP du cœur ou d'un mélange partie à partie de surnageants de broyats auriculaire et ventriculaire est inférieure à la valeur moyenne oreillette + ventricule (12,52) et remarquablement ne correspond pas à la somme des valeurs auriculaire et ventriculaire, fait qui est à rapprocher des résultats obtenus sur le taux de cholinestérase du cœur branchial de *Myxine glutinosa* par Augustinsson *et al.* (1956) ;

3°) le rapport cAMP auriculaire/cAMP ventriculaire = 1 76, ce qui est remarquablement en accord avec le rapport des valeurs respiratoires (Rybak et Boivinet 1959) qui est de 1 75 et avec le rapport mécanique (Jensen 1961) qui est de 1 63.

Nous avons par ailleurs révélé l'existence d'une 3'-5' nucléotide cyclique phosphodiestérase (PDE) tant dans l'oreillette que dans le ventricule de *Myxine glutinosa*. En tenant compte des masses relatives auriculaire et ventriculaire (rapport ~ 1 : 3) les vitesses de décomposition sont les suivantes pendant la première heure d'incubation à 20 °C :

| | |
|---|---|
| — pour le cœur entier | 64 pmol cAMP/mg tissu/min |
| — pour l'oreillette seule | 77 pmol cAMP/mg tissu/min |
| — pour le ventricle seul | 60 pmol cAMP/mg tissu/min |

Ainsi la PDE auriculaire apparait plus active que la PDE ventriculaire

Les cinetiques de vieillissement a 0° C donnent

— pour le cœur entier (dans le 2 premieres heures) de l ordre de 3 54 nmol cAMP/mg tissue essore/h

— pour l oreillette (pendant 3 h) de l ordre de 5 nmol cAMP/mg tissu essore/h

— pour le ventricule (pendant 3 h) de l ordre de 3 nmol cAMP/mg tissu essore/h

## Discussion

*Sur le plan biochimique* nous constatons que l oreillette est a la fois plus riche en cAMP et possede une PDE specifique plus active que le ventricule il en resulte que l oreillette doit posseder un systeme adenyl cyclase plus actif que le ventricule Ceci nous amene a souligner un point important de l enzymologie analytique a savoir que dans des homogenats et les surnageants correspondants ce que l on dose comme substrat est toujours le resultat d une *optimalisation* entre l enzyme de biosynthese (ou enzyme d entree) et l enzyme hydrolytique (ou enzyme de sortie) *sur le plan physiologique* comme l indique le tableau I recapitulatif ci dessous nos presents resultats s y ajoutant on explique comment l oreillette est le « *pacemaker* » *dominant* du cœur branchial de *Myxine* qui possede (Rybak 1960) de multiples centres automatogenes ventriculaires

Il est evident que le processus automatogene de par sa periodicite implique une

Tableau I

| Réferences | | Oreillett | | Ventricule | |
|---|---|---|---|---|---|
| Rybak et Boivinet 1959 | glycogene (µg/poids essoré) | 5 06 ± 1 | | 15 3 ± 1 7 | |
| | acide lactique (d.sous P de la solut on de Young) | 0 0947 | | 0 266 | |
| | resp ration (µl /h/µg P total) | 0 488 ± 0 055 | | 0 277 ± 0 039 | |
| Jensen 1961 | acti ité meca nique (cœu total/ entri cule) | | 1 63 1 | | |
| | | Adrénaline | Noradrenal ne | Adrénal ne | Nor adrenaline |
| Bloom *et al* 1962 | µg catechol amine/g | 13 | 47 (Σ = 60) | 49 | 6 ? (Σ = 55 2) |
| | | | *Cœu t tal* | | |
| Augustinsson *et al* 1956 | cholinestérase (µ mol substrat /30 min/g) | 10 5 | 9 1 | 7 6 | |

Acta physiol scand 1974 90 505—508

From the Cardiorespiratory Research Unit and the Departments of Paediatrics and Physiology University of Turku Finland

# Hemodynamic Effects of Chloralose and Propranolol in Dogs

By

Lauri Halkola Antti Koivikko and Esko Länsimies

Received 22 August 1973

## Abstract

Halkola L. A Koivikko and E Lansimies *Hemodynamic effects of chloralose and propranolol in dogs* Acta physiol scand 1974 90 505—508

Heart rate mean arterial pressure cardiac output, and stroke volume (dye dilution [illegible]) were determined in 10 awake dogs Then the animals were given propranolol and [illegible] 5 dogs were $\beta$ blocked before $\alpha$-chloralose and 5 dogs received the drugs in reverse [illegible] initial responses to the administration of the drugs were insignificant. The final card[illegible] of dogs receiving $\alpha$-chloralose first was lower than that of dogs receiving the same [illegible] reverse order The difference was due to (central or peripheral) $\alpha$ chloralose [illegible] weakende the cardiovascular ability to compensate a decrease of cardiac outp[illegible] observed that $\beta$ blocking prevented and abolished the convulsions and tachyp[illegible] $\alpha$ chloralose

Alfa chloralose has been used for experimental animal anesthesia [illegible] years (Balis and Monroe 1964) There are many studies about [illegible] effects in dogs The results of these studies are however contrad[illegible] 1932 Wiggers 1944 Shabetai Fowler and Hurlburt 1963 Croft [illegible] Buckley 1966 Arfors Arturson and Malmberg 1971 Duchene [illegible] 1972) In neonatal lambs it has previously been observed that the [illegible] system responded with acceleration of heart rate (Koivikko [illegible]

The response of hypothermic dogs and neonatal lambs [illegible] $\alpha$-chloralose anesthesia was different from responses after [illegible] (Koivikko and Lansimies 1972 Halkola Koivikko and Lansi[illegible] 1972) [illegible] was initiated in order to find an answer to the following [illegible]

1 What are the responses of the dog to $\alpha$-chloralose adm[illegible] with propranolol or to both?

2 What might be the regulatory mechanism controlling the observed [illegible]

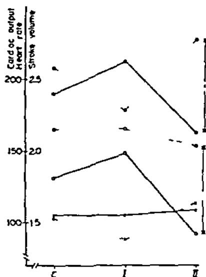



Fig 1 The means of cardiac output (●) in ml/min kg stroke volumes (O) in ml/beat kg and heart rates (×) in beats/min in group A (——) and B ( ) The significance between the 2 groups is indicated by asterisks ($p < 0.001$ = *** $p < 0.01$ = **) C = control situation I and II = first and second administration of the drugs (see text)

## Materials and methods

10 healthy young adult mongrel dogs (mean weight 8 9 kg) from ? litters bred in our laboratory were used in this study The control registrations were performed when animals were unanesthetized and fully conscious and calm. Systemic arterial mean pressure and heart rate were recorded from the descending aorta through a large tube applied through the femoral artery under local anesthesia. Cardiac output and stroke volume were registered by dye-dilution technique described in a previous publication (Halkola *et al* 1972) After control registrations the animals were divided in 2 groups of 5 dogs At the first stage group A received α-chloralose (Merck USA 60 mg/kg b w i v) and group B was β blocked by i v administration of propranolol (Inderal I C I 0 4 mg/kg b w) The effects of injections were continuously followed and the behaviour of the dog was observed (consciousness jerks shivering etc) Registrations were done in triplicate 10—15 min after the injections when all the parameters were stabilized. Then the animals were given the other drug (group A received propranolol and group B α chloralose The doses were the same as in stage one) Again the effects were monitored After the stabilization the same registrations were repeated The means and standard errors of the means were calculated The statistical significance of the changes from control to the first stage and then to the second stage as well as the differences between the means in groups A and B in all registration situations were tested using Student s t test.

## Results

The results are given in Fig 1

*Heart rate* decreased insignificantly ($p > 0.05$) after the β blocking and did not change after α-chloralose In group B the administration of α chloralose caused an acceleration of heart rate ($p < 0.001$) In group A propranolol administration did not change the heart rate

*Systemic arterial mean pressure* remained stable through all (range 110—125 mmHg) stages of these experiments

*Cardiac output* decreased insignificantly after β blocking and increased insignificantly after α chloralose In group B the α chloralose increased cardiac output insignificantly but in group A β blocking caused a rise in cardiac output ($p < 0.05$) The cardiac outputs between groups A and B differed by nearly 40 per cent ($p < 0.001$) in the second stage

*Stroke volume* did not change after β blocking but increased slightly but insignificantly after α-chloralose In the second stage stroke volume decreased insignificantly in both groups The difference between groups A and B was however 35 % ($p < 0.01$)

Administration of α-chloralose alone caused tachypnea shivering and convulsions These phenomena were abolished by the administration of propranolol These phenomena were not observed in animals given propranolol first

## Discussion

Our results agree with those of Bergamaschi *et al* (1971) They concluded that in unanesthetized dogs the β blocking has no effect on the heart rate and cardiac output with doses similar to those used in our study Cardiovascular responses to β blocking in anesthetized dogs are different from and not comparable to those in unanesthetized dogs (Bergamaschi *et al* 1971) The influences of α-chloralose on the hemodynamic parameters were small and insignificant When comparing the two groups receiving the same drugs in different order some confusing results emerge Group B had significantly higher cardiac output and stroke volume than animals in group A The reasons for these observations can be speculated as follows

α-chloralose abolishes the cardiovascular reaction to counteract a decrease in cardiac output

α-chloralose inhibits the myocardial contractility and thus the cardiovascular system cannot compensate the decreased cardiac output by increased stroke volume The administration of α chloralose inhibits (blocks?) myocardial protective capabilities and thus propranolol might directly depress the myocardium resulting in decreased stroke volume and cardiac output while the heart rate remains unchanged

In group B the α chloralose administration accelerated the heart rate resulting in increased cardiac output because the stroke volume remained stable β blocking does not inhibit the supposed receptor like mechanism of α chloralose On the contrary α-chloralose changed the myocardial contractility resulting in clear cut propranolol effect on the stroke volume

The effect of α-chloralose may be central even cortical in nature (Balis and Monroe 1964) Tachypnea convulsions and shivering might be of central origin β blocking abolished these phenomena

α-chloralose cannot be considered as an anesthetic agent for the dog but rather as a hypnotic one

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Acta physiol scand 1974 90 509—512
rom the Zoophysiological Laboratory Department of Zoology University of Turku Finland

# Lowered Sensitivity to Acetylcholine in Hearts from Cold-Acclimated Rats and Frogs

By

M N E Harri and R Tirri

Received 24 August 1973

## Abstract

Harri M N E and R. Tirri *Lowered sensitivity to acetylcholine in hearts from cold acclimated rats and frogs* Acta physiol scand 1974 *90* 509—512

The sensitivity of the heart to the negative chronotropic effect of acetylcholine (ACh) was measured *in vivo* from denervated hearts of the rat and frog before and after they were acclimated to cold In the frog the possible seasonal changes in sensitivity were al o observed In the rat, cold acclimation lowered the sensitivity to ACh This change was significant already at 3 days after cold exposure Cold acclimation lowered the sensitivity to ACh also in the frog but only during the winter On the other hand summer frogs were much more sensitive to ACh than winter frogs It was concluded that it is the increased parasympathetic activity in the cold acclimated rat and in the winter frog which results in the lowering of sensitivity to ACh

The increased sympathetic activity in cold exposed animals leads to increased metabolic sensitivity to catecholamines both in mammals (Carlson 1966) and in amphibians (Harri and Hedensteam 1972) Some authors have also observed an increase in cardiovascular sensitivity to noradrenaline (LeBlanc 1960 Heroux 1961 Evonuk and Hannon 1963) whereas some have found no change (Himms Hagen and Mazurkjevicz Kwilecki 1970) or a decreased sensitivity (Honda *et al* 1962 Tirri *et al* 1973)

On the other hand the parasympathetic system is also much more active in cold acclimated animals since it keeps the activated sympathetic system in balance (LeBlanc and Cote 1967) How this situation changes the ensitivity of cholinergic receptors is not known In the present study the effect of cold acclimation on the sensitivity of the heart to acetylcholine (ACh) was investigated in the rat and in the frog Because in amphibians the parasympathetic activity also shows marked seasonal variation (Miller and Mizell 1972) it was of interest to elucidate whether or not this variation results in an altered sensitivity to ACh

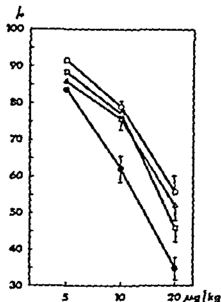



Fig 1

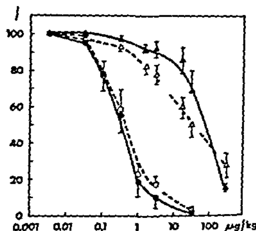



Fig 2

Fig 1 The log dose response curves for the negative chronotropic response to ACh in the control rats kept at 23 C (●) and in the rats acclimated to 5 C for 3 (△) 7 (□) or 30 (○) days The data give the values of the maximal responses, which were at about 7 s after the injections 13 to 19 animals in each group Vertical bars indicate ± S E 100 % = the heart rate before the injections.

Fig 2 The log dose response curves for the negative chronotropic response to ACh in the winter (triangles) and summer (circles) frogs acclimated at 25 C (open symbols) and at 5 C (solid symbols) The responses were measured at 23 C. 10 to 11 animals in each group Other explanations as in Fig 1

## Material and methods

*Adult male Sprague Dawley rats weighing 237—394 g were acclimated to 5 C for 3 7* and 30 days. The control animals were kept at 23 C The rats were anesthetized with 50 mg/kg of pentobarbital s c ACh in the form of chloride was injected into the femoral vein and the heart rate was measured *in situ* from denervated hearts using an electrocardiograph as described earlier Tirri *et al* 1973

Adult male winter (December—January) and summer (June) frogs (*Rana temporaria*) 30—45 g were acclimated to 25 C and to 5 C for at least 2 weeks before use The animals were pitched ACh was injected into the abdominal vein as shown previously by Tirri *et al* (1973) and the responses of the heart were recorded on a smoked drum kymograph with help of an isotonic lever

The doses of ACh are expressed in terms of free drug Student's t test was used in the statistical evaluation of the differences

## Results

The basic heart rate which was 336 ± 2 5 beats/min in the control rats was increased by 3 5 % after 7 days and returned to the initial level after 30 days of prolongde cold exposure (Harri *et al* 1973) When injected into the femoral vein ACh elicited marked bradycardia The maximal effect was reached in 7 s and the effect

was already over in 15 s after the injection With all the doses used the bradycardia caused by ACh was strongest in the control rats (Fig 1) Already at 3 days after cold exposure the lowering of the heart rate caused by doses of 10 $\mu$g/kg ($P < 0.05$) and 20 $\mu$g/kg ($P < 0.01$) of ACh was significantly less than that in the control animals Changes due to further cold acclimation were only small

The basic heart rates measured at 23° C were 29 6 ± 1 9 and 25 6 ± 1 0 beats/min for the 25° C and 5° C acclimated winter frogs respectively For the summer frogs the corresponding values were 28 7 ± 1 3 and 32 3 ± 2.5 beats/min The values of cold and warm acclimated animals do not differ significantly from each other at any season When injected into the abdominal vein ACh elicited its effect on the heart rate immediately and the effect lasted from 30 s to several min depending on the dose The results in Fig 2 show that in the summer frogs the dose response curves for the negative chronotropic response to ACh are very significantly to the left from those of the winter frogs This means that the heart rate of the summer frogs was much more sensitive to ACh The results also show that during the winter bradycardia caused by 18 $\mu$g/kg of ACh was significantly ($P < 0.02$) less in the cold acclimated animals Thus cold acclimation lowered the sensitivity of the heart s cholinergic receptors in winter frogs However in the summer frogs temperature acclimation had no influence on the heart s chronotropic sensitivity to ACh

## Discussion

In the rat and also in the winter frogs cold acclimation lowered the negative chronotropic sensitivity of the heart to ACh The parasympathetic activity is also elevated in cold acclimated animals (LeBlanc and Cote 1967) which suggests that elevated parasympathetic activity may be responsible for the decreased sensitivity of cholinergic receptors This accords with the general concept that subsequent exposure of the effector to the transmitter reduces the number of receptors (Collier 1966) and vice versa (*e g* denervation supersensitivity) However in the summer frogs cold acclimation did not cause subsensitivity to ACh This is consistent with the observation that in contrast to winter frogs the compensatory changes of various metabolic and endocrine functions induced by cold exposure are also lacking in summer animals (Harri and Hedenstam 1972 Lagerspetz *et al* 1973)

Recently it has been found that in the frog the parasympathetic activity is greatly reduced during the summertime the opposite being the case for the sympathetic system (Miller and Mizell 1972) This supports the view stated above that the supersensitivity to ACh in hearts from summer frogs is a consquence of their low parasympathetic activity Moreover Holzapfel (1937) reported that in winter frogs stimulation of the vagus failed to cause bradycardia an indication of the lowered sensitivity of the heart s cholinergic receptors On the other hand the sensitivity of the skeletal muscle to electrical stimulation is also decreased in winter frogs (Holzapfel 1937) This indicates that the lowered sensitivity of cholinergic receptors is not limited only to parasympathetically innervated tissues Thus it is possible that various

endocrine functions also participate in controlling the seasonal changes of the sensitivity of cholinergic receptors.

The lowered sensitivity to ACh is of great physiological importance at low temperatures and in winter conditions since it protects the animals from excessive reduction of the heart work output as a result of vagal reflexes which are required to keep the activated sympathetic system in balance. The rapid development and continuity of this phenomenon, as observed in the rat, further supports its adaptive importance.

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Acta physiol scand 1974 90 513—521

From the Department of Physiology Karolinska Institutet Stockholm Sweden

# On the Mechanism of the Enhancement by Smooth Muscle Stimulants of the Motor Responses of the Guinea Pig Vas Deferens to Nerve Stimulation

By

NILS O SJOSTRAND and GORAN SWEDIN

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## Abstract

SJÖSTRAND N O and G SWEDIN *On the mechanism of the enhancement by smooth muscle stimulants of the motor responses of the guinea pig vas deferens to nerve stimulation* Acta physiol scand 1974 *90* 513—521

Low concentration of various agonists acetylcholine adrenaline noradrenaline histamine serotonin barium chloride bradykinin eledoisin substance P and angiotensin enhance the motor responses of the nerve stimulated guinea pig vas deferens considerably A similar effect can also be obtained by prostaglandins $E_1$ and $E_2$ although these drugs generally depress the responses of the nerve stimulated vas deferens By a comparison with the actions of the agonists on the contractions of nerve stimulated vasa deferentia and on contractions of electrically stimulated denervated or tetrodotoxin paralyzed vasa deferentia it is concluded that with the exception of angiotensin and the prostaglandins the agonists have their main action post junctionally on the smooth muscle cells Angiotensin has mainly a prejunctional action but acts also in part postjunctionally The prostaglandins have a depressant prejunctional action but a stimulant postjunctional action

The guinea pig vas deferens is rather insensitive to exogenous agonists and high concentrations of stimulants have to be given in order to obtain clearcut motor responses of the organ (Sjostrand 1961) However low concentrations of biogenic amines and other smooth muscle stimulants produce conspicuous enhancement of the motor responses of the vas deferens to nerve stimulation (Sjostrand 1961 and later authors *cf* Sjostrand and Swedin 1968 Sjostrand 1973 a) This effect of the stimulants has been suggested to be mainly due to an action on the smooth muscle cells (Sjöstrand and Swedin 1968) but since most of the agonists (acetyl choline (ACh) adrenaline (A) noradrenaline (NA) histamine (Hi) serotonin (5 HT) bradykinin substance P angiotensin and barium) also have neuronal actions (*cf* Sjostrand and Swedin 1968) an effect on the neurotransmission has not been ruled out In fact such an action has been proposed for angiotensin (Benelli Della Bella and Gandini 1964) and ACh (Euler 1970)

The present investigation was performed in order to obtain information on the site of action of the agonists It deals with a comparison of motor responses due to nerve stimulation and responses due to direct electrical stimulation of the smooth muscle cells in denervated or Tetrodotoxin (TTX) paralyzed vasa deferentia

## Methods

38 mottled guinea pigs weighing 400—800 g were used The animals were anesthetized with pentobarbitalsodium (30 mg/kg) The abdomen was opened with a midline incision The vas deferens on one side was gently dissected free Two ligatures (about 2 mm apart) were tied around the vas deferens and its supplying nerves and vessels below the location of the peripheral ganglia (*cf* Sjostrand 1965 Ferry 1967) Denervation was performed by vasotomy between the two ligatures After section of the vas deferens the abdomen was closed The unilaterally denervated guinea pigs were killed by a blow on the head 6—12 days after the operation The vasa deferentia were removed and placed between two parallel platinum electrodes (10 mm long and 8 mm apart) in organ baths (20 or 50 ml) containing Tyrode solution aerated with 6 5 % $CO_2$ in $O_2$ and kept at 37 C The organs were stimulated by field stimulation (Birmingham and Wilson 1963) with square wave pulses (2 ms duration in innervated preparations and 40 ms in denervated or TTX paralyzed preparations) The frequency was 3—10 Hz and stimulation was applied for 5 s every min Supramaximal voltage (20—25 V) was applied for nerve stimulation for smooth muscle stimulation the maximal possible voltage (30 V) of the stimulator (Grass S4) set was used but it was not supramaximal The mechanical responses were recorded isotonically by means of a Harvard heart/smooth muscle transducer coupled to a Grass polygraph Experiments were performed simultaneously on denervated vasa deferentia and the corresponding contralateral control organs The denervation was checked by determination of the catecholamine content of the organs after finishing the experiment (for details see Swedin 1971 a) and by determination of the stimulus duration necessary for eliciting a response The NA content of the denervated organs was 0 00—0 33 $\mu$g/g and that of the controls 9 93—18 26 $\mu$g/g No denervated or TTX paralyzed organ responded to electrical stimulation unless the pulse duration exceeded 5 ms

The Tyrode solution had the following composition (concentrations in mM) NaCl 136 7 KCl 2 7 $CaCl_2$ 1 8 $MgCl_2$ 0 5 $NaHCO_3$ 11 9 $NaH_2PO_4$ 0 4 and glucose 5 5

The following drugs were used acetylcholine bromide l adrenaline bitartrate l nor adrenaline bitartrate histamine hydrochloride serotonin creatinine sulphate angiotensin amide (Hypertensin CIBA) bradykinin (BRS 640 Sandoz) eledoisin (ELD 950 Sandoz) synthetic substance I (gift from Dr S Leeman) $PGE_1$ PGE barium chloride hexa methonium bromide and tetrodotoxin (Sankyo) The concentrations of the amines are given as salts

## Results

All innervated preparations responded with fairly stable contractions to nerve stimulation at stimulation frequencies in the range 5—10 Hz In some preparations it was possible to get stable responses with frequencies as low as 3—4 Hz In order to keep the stimulation frequency within the physiological limits (Folkow 1952) the lowest frequency giving stable responses was purposely used in each individual experiment Innervated preparations could be used for at least 6 h

At stimulation frequencies of 4—10 Hz denervated or TTX paralyzed preparations gave fairly steady responses to direct smooth muscle stimulation during the first 2 h whereafter a successive decline of the responses started Denervated organs could rarely be used for a longer time than 4 h If the stimulation frequency exceeded 10 Hz or the duration of the pulses exceeded 40 ms the preparations rapidly deteriorated possibly due to the electrolytic effect of the stimulation on the organ

bath fluid Denervated preparations often showed a slight tendency to spontaneous activity and changes in basal tone This was rarely observed in innervated preparations Direct smooth muscle stimulation rarely gave contractions of the same magnitude as those due to nerve stimulation

When the actions of the smooth muscle stimulants were compared on the preparations the procedure was the following A concentration of the agonist increasing the responses to nerve stimulation of the control organ to approximately the double was chosen This concentration was then tested on the denervat d preparation which was stimulated with the same frequency as the control In the TTX experiments the agonists were first tested on the responses to nerve stimulation whereafter TTX ($5 \times 10^{7}$ g/ml) was added to the bath After development of full paralysis (indicated by complete resistance to pulses of 2 ms duration and frequencies up to 50 Hz) direct mooth muscle stimulation with pulses of 40 ms and the same frequency as before paralysis was started The same concentrations of the agonists used before paralysis were then tested

The postganglionic nature of the nerve stimulation was checked by addition of hexamethonium bromide ($1 \times 10^{4}$ g/ml) to the bath

*Biogenic amines*

In order to obtain clearcut contractions of the vasa deferentia very high concentrations of the biogenic amines had to be used In nearly all of the preparations it was impossible to obtain contractions by added agonists which matched or exceeded those of the nerve stimulation unless extreme concer rations of the agonists ($>1 \times 10^{4}$ g/ml) were used The minimal concentration for obtaining a clear contraction (about 1/3rd of that due to nerve stimulation at 5 Hz) was in the most sensitive preparation $5 \times 10^{7}$ g/ml for ACh $1 \times 10^{6}$ g/ml for A and NA, $5 \times 10^{6}$ g/ml for Hi and $2 \times 10^{5}$ g/ml for 5 HT In the other preparations at least 10 times higher concentrations has to be added The denervated pr parations were in general more sensitive to added agonists especially A and NA However, because of the great variance in sensitivity to the amines and the irregularity of the shape of the responses in different preparations (Fig 1—4) it was not possible to compare differences in sensitivity to the agonist with a dose respon e analysis Nor was it possible to evaluate the degree of a possible denervation supersensitivity

Concentrations of the biogenic amines being 10—1000 times smaller ($1 \times 10^{9}$ – $1 \times 10^{6}$ g/ml) than those giving clear contraction of the organs exerted a marked enhancement of the motor responses to nerve stimulation As with the direct contractile responses to the agonists there were rather gr a differences in sensitivity among the preparations In general the biogenic amines could be ranked in the following order ACh > A NA > Hi > 5 HT (Fig 1—4) In the mo t sensitive preparation the lowest amine concentration exerting an enhancem t of the motor responses to nerve stimulation was $1 \times 10^{9}$ g/ml for ACh $5 \times 10^{9}$ g/ml f A and NA $2 \times 10^{7}$ g/ml for Hi and $1 \times 10^{6}$ g/ml for 5-HT In the rest of the prepara tions the threshold concentrations were 5—10 times the

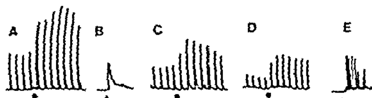



Fig. 1. Isolated guinea pig vas deferens. *A* Nerve stimulation (25 V, 2 ms, 4 Hz, 5 s every min) at dot acetylcholine $5 \times 10^{-9}$ g/ml. *B* Contraction by acetylcholine 2×10 g/ml. *C* Smooth muscle stimulation (30 V, 40 ms, 4 Hz, 5 s every min) after TTX ($5 \times 10^{-7}$ g/ml) at dot acetylcholine $5 \times 10^{-9}$ g/ml. *D* Contralateral denervated organ smooth muscle stimulation (30 V, 40 ms, 4 Hz, 5 s every min) at dot acetylcholine $5 \times 10^{-9}$ g/ml. *E* Denervated organ at dot acetylcholine $2 \times 10^{-6}$ g/ml.

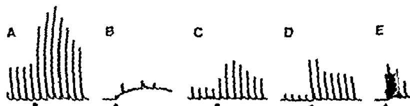



Fig. 2. Isolated guinea pig vas deferens. *A* Nerve stimulation (25 V, 2 ms, 5 Hz, 5 s every min) at dot adrenaline $2 \times 10^{-7}$ g/ml. *B* Adrenaline $5 \times 10^{-6}$ g/ml. *C* Smooth muscle stimulation (30 V, 40 ms, 5 Hz, 5 s every min) after TTX (5×10 g/ml) at dot adrenaline 2×10 g/ml. *D* Contralateral denervated organ smooth muscle stimulation (30 V, 40 ms, 5 Hz, 5 s every min) at dot adrenaline $2 \times 10^{-7}$ g/ml. *E* Denervated contralateral organ at dot adrenaline $5 \times 10^{-6}$ g/ml.

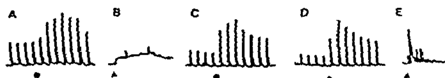



Fig. 3. Isolated guinea pig vas deferens. *A* Nerve stimulation (25 V, 2 ms, 5 Hz, 5 s every min) at dot histamine $1 \times 10^{-6}$ g/ml. *B* Histamine $1 \times 10^{-4}$ g/ml. *C* Smooth muscle stimulation (30 V, 40 ms, 5 Hz, 5 s every min) after TTX (5×10 g/ml) at dot histamine 1×10 g/ml. *D* Denervated contralateral organ stim. as in C at dot histamine $1 \times 10^{-4}$ g/ml. *E* Denervated organ at dot histamine $1 \times 10^{-4}$ g/ml.

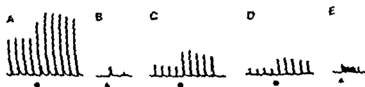



Fig. 4. Isolated guinea pig vas deferens. *A* Nerve stimulation (25 V, 2 ms, 5 Hz, 5 s every min) at dot serotonin $5 \times 10^{-6}$ g/ml. *B* Serotonin $5 \times 10^{-4}$ g/ml. *C* Smooth muscle stimulation (30 V, 40 ms, 5 Hz, 5 s every min) after TTX ($5 \times 10^{-7}$ g/ml) at dot serotonin $5 \times 10^{-6}$ g/ml. *D* Contralateral denervated organ smooth muscle stimulation (30 V, 40 ms, 5 Hz, 5 s every min) at dot serotonin $5 \times 10^{-6}$ g/ml. *E* Denervated organ serotonin $5 \times 10^{-4}$ g/ml.

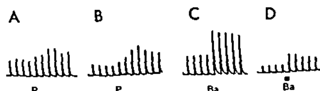



Fig 5 Isolated guinea pig vas deferens *A* Nerve stimulation (25 V ? ms 4 Hz 5 s ev min) at P Substance P 5 × 10 ⁸ g/ml *B* Contralateral denervated organ stimulation 30 V 4 ms 40 Hz 5 s ev min at P Substance P 5 × 10 ⁸ g/ml *C* Nerve stimulated vas deferens (25 V 2 ms 5 Hz 5 s ev min) at Ba barium chloride 1 × 10 ⁵ g/ml *D* Contralateral denervated organ (30 V 40 ms 5 Hz 5 s ev min) at Ba barium chloride 1 × 10 g/ml

Since it has been reported that A and NA can exert an inhibition of the motor response of the guinea pig vas deferens to nerve stimulation (Large 1965 Hotta 1969 Ambache and Zar 1971) attention was given to this possibility although the scope of the investigation was to study the potentiation of the agonists In 15 preparations NA and A was added successively in concentrations from $1 \times 10^{10}$ to $1 \times 10^{5}$ g/ml Except for 1 preparation all preparations responded with an enhancement of the response to nerve stimulation appearing at concentrations of $1 \times 10^{8}$—$1 \times 10^{7}$ g/ml the potentiation persisted up to $1 \times 10$ g/ml where huge contractions due to nerve stimulation were superimposed on the contraction due to the agonist In one preparation the first contraction due to nerve stimulation after addition of A or NA was reduced at $1 \times 10^{7}$ and $1 \times 10^{6}$ g/ml but the following contractions were enhanced After addition of propranolol ($1 \times 10^{7}$ g/ml) this preparation responded with enhancement of all the responses after addition of A or NA In this case the corresponding denervated preparation showed the same pattern *i e* the first contraction after A or NA was reduced but the others were enhanced After propranolol all responses were enhanced

In nearly all experiments the biogenic amines increased the responses of directly stimulated *TTX paralysed vasa deferentia* to the same extent as previously the responses to nerve stimulation *i e* a concentration of the biogenic amines approximately doubling the responses to nerve stimulation also doubled those due to direct smooth muscle stimulation (Fig 1—4 c)

Similar results as with direct smooth muscle stimulation of TTX paralysed organs were obtained with the *denervated vasa deferentia* (Fig 1—4 d) although the enhancement by the biogenic amines of the motor responses to electrical stimulation was often greater in these preparations than in the corresponding control organs (Fig 2 d and 3 d)

Fig 1—4 illustrate the typical results obtained by the biogenic amines The responses to nerve stimulation and the potentiation of these responses by a low concentration of the agonist is shown in a) The contraction induced by a high concentration of the agonist is shown in b) In c) and d) the responses due to direct smooth muscle stimulation of TTX paralysed (c) and the denervated preparation (d) is shown and in e) the contraction induced by a high concentration of the agonist on the denervated preparation is shown

*Polypeptides*

Distinct enhancements of the moto ... of the vas deferens to ner

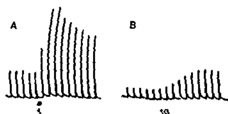



Fig 6 Isolated guinea pig vas deferens *A* Nerve stimulation (25 V 2 ms 5 Hz 5 s ev min) at *I* angiotensin $1 \times 10^{-8}$ g/ml *B* Contralateral denervated organ stimulation (30 V 40 ms 5 Hz 5 s ev min) at *10* angiotensin $1 \times 10^{-5}$ g/ml

lation were obtained by *bradykinin* ($0.5$—$2 \times 10^{-8}$ g/ml), *eledoisin* ($1$—$4 \times 10^{-8}$ g/ml) and synthetic *substance P* ($0.1$—$1 \times 10^{-7}$ g/ml) The degree of enhancement was the same or greater in preparations subjected to direct electrical stimulation of the smooth muscle In Fig 5 a and b records from an experiment with substance P are presented

*Angiotensin* differed from the other polypeptides, a marked potentiation of the responses to nerve stimulation was obtained with concentrations of $0.5$—$2 \times 10^{-8}$ g/ml In order to obtain the same degree of enhancement in denervated or TTX paralysed preparations the angiotensin concentration had to be increased at least 10 times (Fig 6 a & b)

*Barium*

Barium chloride ($1$—$5 \times 10^{-5}$ g/ml) exerted a marked enhancement on responses due to nerve stimulation as well as those due to direct stimulation of the smooth muscle (Fig 5 c d)

*Prostaglandins*

$PGE_1$ and PGE in low concentrations (1—20 ng/ml) suppressed the responses to nerve stimulation in all tested preparations With high concentrations (100—500

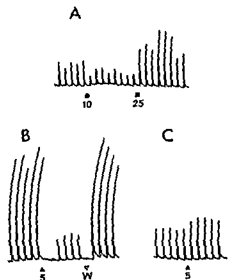



Fig 7 Isolated guinea pig vas deferens *A* Nerve stimulated vas deferens (25 V 2 ms 5 Hz 5 s ev min) at *10* prostaglandin $E_1$ 10 ng/ml at 25 prostaglandin $E_1$ 25 ng/ml *B* Nerve stimulated vas deferens from another animal (25 V 2 ms 7 Hz 5 s ev min) at *5* prostaglandin $E_1$ 5 ng/ml at *W* wash *C* Contralateral denervated preparation stimulation (30 V 40 ms 7 Hz 5 s ev min) at *5* prostaglandin $E_1$ 5 ng/ml

ng/ml) the respon es to nerve stimulation were usually increased frequently after a brisk suppression of the first 1 or 2 responses

In certain preparations the difference between a suppressing concentration of the prostaglandins and a potentiating could be very narrow as illustrated in Fig 7 a. In denervated or TTX paralysed preparations the effect of prostaglandins was an enhancement of the contractions seen in all concentrations from 5 ng/ml up to 500 ng/ml (Fig 7 c)

## Discussion

The present investigation shows that low concentrations of various smooth muscle stimulant enhance the motor response of the vas deferens due to nerve stimulation as well as that due to direct electrical stimulation of the smooth muscle With the exception of angiotensin and the prostaglandins the results obtained with the two types of stimulation are qualitatively almost identical for the different smooth muscle stimulants This indicates that the biogenic amines bradykinin eledoisin substance P and barium chloride increase the responses of the guinea pig vas deferens to nerve stimulation mainly by a direct action on the smooth muscle cells and not by facilitating the transmitter release Furthermore electrophysiological studies have indicated that the main action of the agonists is a partial depolariza tion of the smooth muscle cell membrane which brings the cells closer to the firing level of action potentials and also promotes the propagation of action potentials (Sjöstrand 1973 a) An additional effect on the contractile processes effectuated by *e g* increased Ca influx to the cells or release of membrane bound calcium can of course not be excluded all the more as in smooth muscle depolarization and action potentials appear to be associated with movements of calcium ions (*cf* Bennet 1967 1972 Bulbring and Tomita 1970)

Concerning the action of adrenaline and noradrenaline we have not been able to confirm the results of Ambache and Zar (1971) that the usual effect of the catecholamines is an inhibition of the responses to nerve stimulation which is not due to a $\beta$ effect of the catecholamines In the only experiment where an inhibi tion was noted it was apparently due to stimulation of inhibitory $\beta$ receptors since it was prevented by propranolol The discrepancy between our results and those of Ambache and Zar (1971) can be due to differences in stimulation pattern since they used higher frequencies (up to 50 Hz) and fewer pulses (generally 5) How ever also by adopting their type of stimulation the usual effect of catecholamines on untreated preparations is an enhancement (Sjostrand unpublished observations) This does not disprove that catecholamines could have a none $\beta$ adrenergic in hibitory effect on the responses of the nerve stimulated vas deferens but it does not support the claim of Ambache nad Zar that the neurotransmission in the vas de ferens is nonadrenergic (*for further discussion see Sjostrand 1973 b*)

The finding that angiotensin has a greater effect on the responses to nerve stimu lation than on direct smooth muscle stimulation supports the view that angiotensin

has an action on transmitter release from sympathetic nerves (*cf.* Starke 1971). Angiotensin has, however, also a direct effect on the smooth muscle. In a previous study we found that angiotensin also facilitated ganglionic transmission in pre-synaptically stimulated vasa deferentia (Sjöstrand and Swedin 1968). Thus the sympathetic nerve-vas deferens preparation is one more example of an isolated organ in which angiotensin exerts complex actions.

The inhibition by low concentrations of prostaglandins of the motor responses to nerve stimulation is in accordance with the findings of Euler and Hedqvist (1969, 1972), Ambache and Zar (1971) and Swedin (1971 b). The potentiation of the responses to nerve stimulation seen with higher concentrations of the prostaglandins agrees with the findings of Mantegazza and Naimzada (1965) and Euler and Hedqvist (1969, 1972). The present finding that the responses to direct electrical stimulation of the smooth muscle always are increased by the prostaglandins is a strong support of the conception that the prostaglandins depress the sympathetic neurotransmission (see discussion by Hedqvist and Euler 1972) but has a direct stimulant effect on the smooth muscle of the guinea-pig vas deferens. This concept is further supported by the observation that $PGE_1$ suppresses the excitatory junction potentials in the vas deferens but hypopolarizes the smooth muscle of the organ (Sjöstrand 1972).

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Acta physiol scand 1974 90 522—532
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# 5-HT Storage in Rat and Rabbit Blood Platelets The Separation of ATP-Containing and Sulphomucopolysaccharide-Containing Granules

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## Abstract

ANDERSON P S A SLORACH and B UVNÄS *5 HT storage in rat and rabbit blood platelets the separation of ATP containing and sulphomucopolysaccharide containing granules* Acta physiol scand 1974 90 522—532

Sonicated rabbit and rat platelets were subjected to differential centrifugation and the mixed organelle fraction so obtained then separated by ultracentrifugation on a discontinuous sucrose gradient The distribution curves for 5-hydroxytryptamine (5 HT) histamine (studied in rabbit only) and adenosine 5 triphosphate (ATP) were similar Electron microscopy of the amine-containing fractions revealed the presence of very dense granules. These fractions contained little or no hexosamine or $^{35}S$ two indicators of the presence of sulphomucopolysaccharides (SMPS) A second type of granule α granules was found in fractions corresponding to peaks on the hexosamine and $^{35}S$ distribution curves The observation that in rabbit 5 HT histamine and ATP are localized to the same subcellular fractions as the very dense granules agrees with the results of earlier workers An analogous situation appears to exist in rat platelets The biogenic function of the SMPS containing α granules remains uncertain It might be suggested that they like the mast cell granules utilize their SMPS protein complex for the storage of electrically charged substances

From the results of biochemical ultrastructural and autoradiographic studies on rabbit blood platelets and granule fractions isolated therefrom it has been concluded that 5 hydroxytryptamine (5 HT) (*vide e g* Tranzer *et al* 1966 1968 Da Prada *et al* 1967 Bak *et al* 1967 Solatunturi and Tuomisto 1968 Davis and White 1968) and histamine (Da Prada *et al* 1967) are stored in the so-called very dense granules (VDG) observed in electron micrographs of such cells *Furthermore* it has been demonstrated that such granules also contain adenosine 5 triphosphate (ATP) (Da Prada and Pletscher 1968) and since 5 HT and ATP yield high molecular weight aggregates under certain conditions *in vitro* it has been postulated that the *in vivo* storage of 5 HT in platelets involves micelle formation with ATP (Berneis *et al* 1969 1971)

Recently in a preliminary communication Åborg and Uvnas (1971) reported that rat platelets contain a sulphomucopolysaccharide (SMPS) protein complex capable of binding biogenic amines *in vitro* Furthermore Uvnas and co-workers (Uvnäs *et al* 1970 Bergendorff and Uvnas 1972) have demonstrated that the

histamine and 5 HT present in rat mast cells is ionically bound to an SMPS (heparin) protein complex present in the storage granules and that SMPS protein complexes are also present in amine containing granule fractions isolated from adrenal medulla and adrenergic nerves of various species (Fillion *et al* 1971 Åborg *et al* 1972) The presence of such complexes capable of binding biogenic amines in a variety of biogenic amine containing tissues led Uvnas (1973) to suggest that they may be involved in the *in vivo* binding of biogenic amines

The present combined biochemical and ultrastructural study was undertaken in order to try and establish whether or not such an SMPS protein complex was present in the 5 HT containing granules in rat and rabbit platelets Rat platelets were used so that the results could be correlated with those of Åborg and Uvnas (1971) and rabbit platelets because these cells contain relatively large amounts of 5 HT and methods of separating the amine containing granules have already been described (for references see Methods) In order to facilitate the study of the distribution of small amounts of SMPS the latter were labelled by injecting the animals with Na $^{3}$ $SO_4$ two days prior to sacrifice The distribution of hexosamine a constituent of SMPS was used as a second guide to SMPS distribution

## Methods

In a series of pr liminary experiments using slight modifications of the procedure described by Åborg and Uvnas (1971) (separation of sonicated rat platelet organelles on a continuous 5—10 % Ficoll density gradient in isotonic sucrose) the present authors found that it was possible to achie e some separation of the 5-HT and $^{3}$ S distribution curves *However the* degree of separation was insufficient to enable any definite conclu ions to be drawn and other separation techniques were investigat d Four attempts to separate *a granules and very dense* granules from rabbit platelets using the method of Da Prada and Pletscher (1968) were unsuccessful and this method was abandoned Instead, a modification of the procedure described by Solatunturi and Tuomisto (1968) was used (*vide infra*)

*Rabbit platelets*
Albino rabb ts (2 5—4 5 kg b w ) of either sex were injected s c with 2 mCi of Na $^{3}$ $SO_4$ Two days later they were anesthetized with Nembutal® (30 mg/kg) and ether as required and arterial blood was collected via a carotid cannula and then mixed with 10 % v/v disodium EDTA solution (1 5 % w/v in 0 7 % w/v sodium chloride) and cooled in ice The platelet rich plasma obtained by centrifugation at 300 × *g* for 15 min at 4 C was centrifuged at 000 × *g* for 25 min to precipitate the platelets All g values refer to the maximum gravitational force in the tube

When $^{3}$H 5 HT labelled platelets were required the platelets ere suspended in 15 *ml of* 0 9 % w/v sodium chloride solution containing 25 or 50 μCi of $^{3}$H 5 HT and incubated for 60 min at 37 C with constant shaking

After washing twice in 0 9 % w/v sodium chloride at 4 C the platelets were suspended *in* ice-co d 0 32 M sucrose and 5 ml aliquots were then son cated for 10 in an MSE Ultrason c d sintegrator (100 W model) operating at an amplitude of 4 μm The *sonicate* was c ntrifuged at 3600 × g for 25 m n at 4 C and the resulting supernatant was recentrifuged at 14 462 × *g for* 5 min at 4 C The precipitate obtained in the latter centrifugation was resuspended in 0 32 M sucrose (1—3 ml) and 1 ml aliquots of the *suspension* were layered onto d scont nuous sucrose gradients consisting of 1 ml fractions of 2 6 M 2 0 M 1 4 M and 1 2 M s crose respecti ely and centrifuged at 98 064 × *g* for 60 m n at 4 C *in* a Beckman SW 50L rotor

Aft r centrifugation 5 drop fractions were collected via a hypodermic needle inserted at the base of the tube After collecting the sucrose gradient, any precipitate at the bottom of the tube was removed by *suspending it in* 5 drops of distilled wate The fractions were either collected d rectly *into* vials *for liquid* scintillation counting (double isotope experiments) or diluted with 0 02 N HCl and aliquots then taken for the various assays

Acta physiol scand 1974 90 522—532

From the Department of Pharmacology Karolinska Institutet Stockholm Sweden

# 5-HT Storage in Rat and Rabbit Blood Platelets The Separation of ATP-Containing and Sulphomucopolysaccharide-Containing Granules

By

PER ANDERSON STUART A SLORACH and BÖRJE UVNAS

Received 21 May 1973

## Abstract

ANDERSON P S A SLORACH and B UVNAS *5 HT storage in rat and rabbit blood platelets the separation of ATP containing and sulphomucopolysaccharide containing granules* Acta physiol scand 1974 *90* 522—532

Sonicated rabbit and rat platelets were subjected to differential centrifugation and the mixed organelle fraction so obtained then separated by ultracentrifugation on a discontinuous sucrose gradient The distribution curves for 5 hydroxytryptamine (5 HT) histamine (studied in rabbit only) and adenosine 5 triphosphate (ATP) were similar Electron micro copy of the amine containing fractions revealed the presence of very dense granules These fractions contained little or no hexosamine or $^{35}S$ two indicators of the presence of sulphomucopolysaccharides (SMPS) A second type of granule *a* granules was found in fractions corresponding to peaks on the hexosamine and $^{35}S$ distribution curves The observation that in rabbit 5 HT histamine and ATP are localized to the same subcellular fractions as the very dense granules agrees with the results of earlier workers An analogous situation appears to exist in rat platelets The biogenic function of the SMPS containing *a* granules remains uncertain It might be suggested that they like the mast cell granules utilize their SMPS protein complex for the storage of electrically charged substances

From the results of biochemical ultrastructural and autoradiographic studies on rabbit blood platelets and granule fractions isolated therefrom it has been concluded that 5 hydroxytryptamine (5 HT) (*vide e g* Tranzer *et al* 1966 1968 Da Prada *et al* 1967 Bak *et al* 1967 Solatunturi and Tuomisto 1968 Davis and White 1968) and histamine (Da Prada *et al* 1967) are stored in the so-called very dense granules (VDG) observed in electron micrographs of such cells Furthermore it has been demonstrated that such granules also contain adenosine 5 triphosphate (ATP) (Da Prada and Pletscher 1968) and since 5 HT and ATP yield high molecular weight aggregates under certain conditions *in vitro* it has been postulated that the *in vivo* storage of 5 HT in platelets involves micelle formation with ATP (Berneis *et al* 1969 1971)

Recently in a preliminary communication Åborg and Uvnas (1971) reported that rat platelets contain a sulphomucopolysaccharide (SMPS) protein complex capable of binding biogenic amines *in vitro* Furthermore Uvnas and co-workers (Uvnas *et al* 1970 Bergendorff and Uvnas 1972) have demonstrated that the

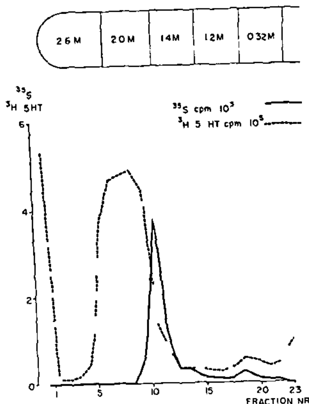



Fig 1 Distribution of $^{3}H$ 5 HT and $^{35}S$ after centrifuging the $14\,462 \times g$ precipitate from sonicated rabbit platelets on a discontinuous sucrose gradient (for details see Methods) The approximate positions of the different sucrose layers in Fig 1—5 is indicated schematically above the distribution curves

In 2 exp s with rat platelets the distribution was $3600 \times g$ precipitate 45 5 % (31—60) $14\,462 \times g$ supernatant 19 5 % (16—23) $14\,462 \times g$ precipitate 34 5 % (23—46)

*Distribution of amines ATP $^{35}S$ hexosamine and protein after centrifugation on the sucrose gradient*

a) *Double isotope studies rabbit* 2 expts were performed using platelets labelled with both $^{3}H$ 5 HT and $^{35}S$ The distribution curves from one such experiment are shown in Fig 1 The $^{35}S$ labelled platelets were incubated with $50 \mu Ci$ of $^{3}H$ 5 HT in this experiment There was a separation of the two types of radioactivity—the tritiated 5 HT was found at the bottom of the gradient and in the 2 M sucrose fraction whereas the $^{35}S$ activity was essentially absent from these fractions and instead located at the interface between the 2 M and 1 4 M sucrose layers

b) *Single isotope studies rabbit* In a further 6 expts the distribution of $^{35}S$ and endogenous 5 HT (6 expts each) ATP (2 expts) protein (1 expts) histamine (1 expt) and hexosamine (1 expt) was studied In each experiment the distribution of $^{35}S$ and 5 HT was similar to that described above in the double isotope

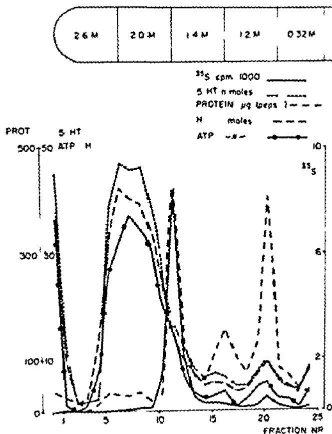



Fig 2 Distribution of 5 HT histamine (Hi), ATP, protein and $^{35}$S after centrifuging the 14 400 × g precipitate from sonicated rabbit platelets on a discontinuous sucrose gradient (for details see Methods). Note the virtual absence of $^{35}$S in the amine-containing peaks at the base of the tube and in the 2.0 M sucrose layer

studies. Although there was a slight overlapping of the $^{35}$S and 5 HT peaks at the 2.0—1.4 M sucrose interface (Fig 2 and 3) it should be noted that the 5 HT peaks at the bottom of the tube and in the 2 M sucrose layer were essentially free from $^{35}$S. The distribution curves for 5 HT, histamine and ATP were found to be very similar (Fig 2). The molar ratio 5 HT/ATP in the amine peaks was found to be 1.5—1.9 and 1.2—1.5 respectively in the 2 expts performed. This is somewhat lower than the ratio (2) reported by Pletscher *et al* (1968). The protein distribution curve (Fig 2) shows that the amine- and ATP-containing layers contain relatively little protein, whereas the $^{35}$S peak contains a much larger amount. Protein peaks were also found at the 1.4—1.2 and 1.2—0.32 M sucrose interfaces.

Hexosamine was found mainly in two peaks—the larger coinciding with the $^{35}$S containing peak and the other lying at the 1.2—0.32 M sucrose interface (Fig 3).

c) *Single isotope studies, rat.* In 3 expts the distribution of $^{35}$S and endogenous 5 HT (3 expts each), ATP (2 expts) and hexosamine (1 expt) was studied. The distribution curves for $^{35}$S, ATP and endogenous 5 HT (Fig 4) were similar to those obtained with rabbit platelets—the only major difference was that the 5 HT and ATP-containing band in the 2.0 M sucrose layer in the rat experiments was concentrated at the bottom of this layer rather than extending over it. This dif

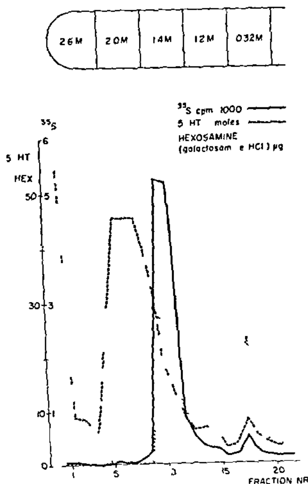



Fig 3 Distribution of 5 HT $^{35}$S and hexosamine after centrifuging the 14 462 × g precipitate from sonicated rabbit platelets on a discontinuous sucrose gradient (for details see Methods)

ference is correlated with the difference in the appearance of the gradients from rabbit and rat experiments after centrifugation (*vide supra*) Although the separation of the 5 HT and ATP containing fractions from the $^{35}$S containing fraction was better than in the rabbit studies the latter fraction was still contaminated with the former

As in the rabbit studies the $^{35}$S peak was found to contain hexosamine (Fig 5) this was the only major hexosamine peak found in the rat experiments

*Electron microscopy*

*Granules isolated from rabbit platelets* The fraction at the 2 0—1 4 M sucrose interface (EM Fraction I) was an almost pure granule fraction (Fig 6 a, b) The vast majority of the granules were moderately electron dense and appeared to be surrounded by a trilaminar membrane which could be seen at favourable planes

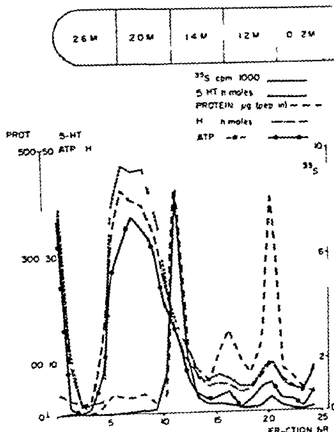



Fig 2 Distribution of 5-HT histamine (Hi) ATP protein and ³⁵S after centrifuging the 14 462 × g precipitate from sonicated rabbit platelets on a discontinuous sucrose gradient (for details see Methods) Note the virtual absence of ³⁵S in the amine-containing peaks at the base of the tube and in the 2 0 M sucrose layer

studies Although there was a slight overlapping of the ³⁵S and 5 HT peaks at the 2 0—1 4 M sucrose interface (Fig 2 and 3) it should be noted that the 5 HT peaks at the bottom of the tube and in the 2 M sucrose layer were essentially free from ³⁵S The distribution curves for 5 HT histamine and ATP were found to be very similar Fig 2 The molar ratio 5 HT/ATP in the amine peaks was found to be 1 5—1 9 and 1 2—1 5 respectively in the 2 expts performed This is somewhat lower than the ratio (2) reported by Pletscher *et al* (1968) The protein distribution curve (Fig 2 shows that the amine and ATP-containing layers contain relatively little protein whereas the ³⁵S peak contains a much larger amount Protein peaks were also found at the 1 4—1 2 and 1 2—0 32 M sucrose interfaces

Hexosamine was found mainly in two peaks—the larger coinciding with the ³⁵S-containing peak and the other lying at the 1 2—0 32 M sucrose interface (Fig 3)

c) *Single isotope studies rat* In 3 expts the distribution of ³⁵S and endogenous 5 HT (3 expts ecah ATP (2 expts) and hexosamine (1 expt) was studied The distribution curves for S ATP and endogenous 5 HT (Fig 4) were similar to those obtained with rabbit platelets—the only major difference was that the 5 HT and ATP-containing band in the 2 0 M sucrose layer in the rat experiments was concentrated at the bottom of this layer rather than extending over it This dif

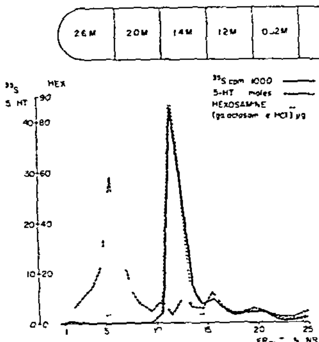
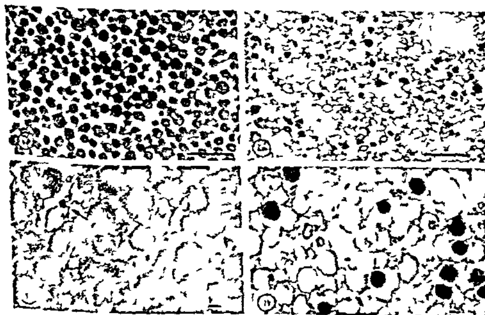



Fig 5 Distribution of 5-HT $^{35}$S and hexosamine after centrifuging the 14 462 × g precipitate from sonicated rat platelets on a discontinuous sucrose gradient (for details see Methods)



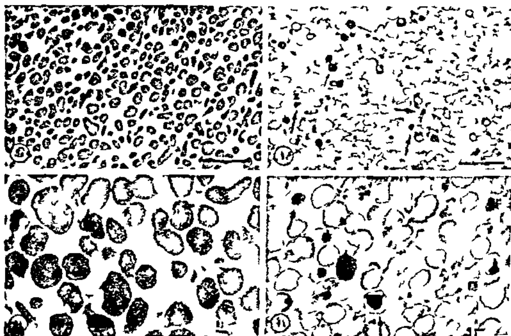


Fig 8 a b Electron micrographs of EM Fraction I (2 0—1 4 M sucrose interface) from rat platelets showing an almost homogeneous fraction of α granules Magnification 8 a 12 000 × 8 b 29 000 ×

Fig 9 a b Electron micrographs of EM Fraction II (band in 2 0 M sucrose layer) from rat platelets showing vesicles the majority of which are empty However many very dense granules can be observed (thick arrows) Some α granules are also present (thin arrows) Magnification 9 a 12 000 × 9 b 29 000 ×

## Discussion

In the present experiments with rabbit platelets it has been demonstrated that the fractions (EM Fractions II and III) which contained the bulk of the 5 HT and histamine also contained ATP relatively small amounts of protein and little or no hexosamine Furthermore these fractions contained the highest proportion of very dense granules The observation that 5 HT histamine and ATP are localized to the same subcellular fractions as the very dense granules agrees with the results of earlier workers (for references see Introduction) In a recent study Da Prada *et al* (1972) reported that 5 HT and ATP containing very dense granule fractions from rabbits guinea pigs and rats contained very small amounts of hexosamine

Fig 6 a b Electron micrographs of EM Fraction I (2 0—1 4 M sucrose interface) from rabbit platelets showing moderately electrondense α granules surrounded by perigranular membranes (thin arrows 6 b) and an occasional very dense granule (thick arrow 6 b) Magnification 6 a, 12 000 × 6 b 29 000 ×

The net like structure seen between granules in the electron micrographs from rabbit is due to the gelatin embedding

Fig 7 a, b Electron micrographs of EM Fraction III (bottom of sucrose gradient) from rabbit platelets *The ultrastructural appearance is very similar to* that of EM Fraction II except that the proportion of α granules appears to be smaller Magnification 7 a, 12 000 × 7 b 29 000 ×

The results of the present studies with rat platelets are analogous to those obtained with rabbit platelets. The amine containing granule fractions from rats showed less very dense material than the corresponding fractions from rabbit. This might be related to the fact that rat platelets contain much less 5-HT than rabbit platelets (0.4 μg compared to 7.5 μg per $10^9$ platelets, Humphrey and Jaques 1954).

The proportion of α granules in the granule fractions from rabbit and rat platelets examined by electron microscopy was related to the distribution of $^{35}$S and hexosamine: the almost pure α granule fraction (FM Fraction I) found at the 2.0—1.4 M sucrose interface corresponded to $^{35}$S and hexosamine peaks on the distribution curves. Anderson and Odell (1958) reported the presence of chondroitin sulphate in rat blood platelets. Åborg and Uvnäs (1971) reported the presence of an SMPS protein complex in granule fractions from sonicated rat platelets. An SMPS protein complex has been demonstrated in α granules isolated from both rabbit and rat platelets using the technique from the present study, and the SMPS has been identified as chondroitin sulphate (Bergqvist *et al.*, to be published).

Although SMPS contain sulphate (which can be labelled by injecting $^{35}SO_4$) and hexosamine residues, both of which can be used as guides to SMPS distribution, both $^{35}$S and hexosamine may separately be present in compounds other than SMPS. Thus the distribution of these marker substances is not necessarily in itself a reliable guide to the distribution of the SMPS. However, the virtual absence of both $^{35}$S and hexosamine in the amine containing fractions from rabbit and rat platelets is evidence against the presence of SMPS there. The presence of a small proportion of α granules in the electron micrographs of the amine containing fractions correlates with the presence of very small amounts of $^{35}$S and hexosamine in these fractions.

In the present study no 5-HT was found in the SMPS containing α granule fraction despite the fact that an SMPS protein complex with a considerable capacity to bind biogenic amines *in vitro* was obtained by water lysis of a granule fraction from sonicated rat platelets (Åborg and Uvnäs 1971).

The biogenic function of the SMPS containing α granules thus remains unclear. If they do not store biogenic amines released from their weak ionic binding during the isolation procedure, they may be involved in the storage of other electrically charged substances, *e.g.* enzymes or blood clotting factors reported to be stored and released by platelets (Holmsen, Day and Stormorken 1969).

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Acta physiol scand 1974 90 533—543

From the Department of Physiology Veterinärhogskolan Stockholm Sweden

# Efferent Chorda Tympani Activity and Salivary Secretion in the Rat

By

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## Abstract

HELLEKANT G and E C HAGSTROM *Efferent chorda tympani activity and salivary secretion in the rat* Acta physiol scand 1974 *90* 533—543

It is known that nerve impulses to the mandibular salivary gland must be carried in the chorda tympani proper nerve although they have never been recorded This study presents recordings of efferent impulses in this nerve and simultaneous observations of secretion from this gland in rats under neuroleptic analgesia, during electrical stimulation of he salivary nuclei in the brainstem Stimulation was found to increase the impulse activity in the nerve and to lead to secretion of saliva The secretion ceased when the nerve was blocked The relationship was best described by a straight line plot of the logarithm of the increased nerve activity against secretion the correlation coefficient being 0 84 Barbiturate abolished the effect of brain stimulation. This indicates that nuclear structures and not chorda tympani fibres were being stimulated

The chorda tympani proper nerve is a branch of the facial nerve It joins the lingual proper nerve during its peripheral course and forms with the latter the chorda lingual nerve (Langley 1898) from which branches are given off to the sublingual and mandibular salivary glands It has long been known that the secretion of these glands is mainly governed by impulse activity in the chorda tympani proper nerve This knowledge is based on anatomical features on the absence of secretion after the nerve has been cut and on the observation of secretion during electrical stimulation of the nerve The actual secretory impulses have never been recorded (Emmelin 1967) and so all conclusions concerning these impulses have been based on indirect observations such as comparisons between the secretion observed during feeding in the unanesthetized animal and the secretion obtained during electrical stimulation of the chorda tympani proper in the same animal under anesthesia (Emmelin 1967)

Since the technique developed at this laboratory (Hellekant 1971) *offers* a possibility of studying the actual impulse traffic in the chorda tympani proper

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nerve it seemed of interest to attempt a study of the relationship between the actual impulses which are known to exist in the nerve and the secretion they may evoke

## Methods

25 male Sprague Dawley rats weighing 270—330 g were used Hypnorm® (Leo) was used as the initial anesthesia according to the procedure described earlier (Hellekant 1971 a) When additional anesthesia was necessary Leptanal® (Leo) was used It contains only the narcotic constituent of the Hypnorm not the tranquillizer Gallamin iodide was used as muscular relaxant and the animals were then artificially ventilated For reasons to be described later some animals were anesthetized with Mebumal® 1 ml of which contains 60 mg pentobarbital sodium

One femoral vein and one femoral artery were always cannulated to allow iv injections and recording of blood pressure The rectal temperature was maintained at 37±1 C The further surgical preparation was carried through in three steps First the right mandibular salivary duct was cannulated and the secretion checked in the manner described earlier (Hellekant and Kasahara 1973 b) Then the animal was mounted in a special stereotaxic instrument which does not use ear bars A small hole was drilled through the skull on the borders of the parietal and interparietal bones approximately 8 mm caudal and 1 7 mm lateral of the bregma. By means of a micromanipulator type Lundberg the electrode was placed in that region of hindbrain which gave a salivary flow of about 30 mg/30 s to a 20 Hz stimulation frequency In most cases this was done in a single penetration A 27 gauge needle with an inserted stainless steel wire served as the stimulation electrode The electrode was then fixed to the skull with acrylic plastic When this was done the animal was moved from the stereotaxic instrument into the usual headholder after which the chorda tympani proper was dissected free

*Recordings were made from the intact chorda tympani proper nerve The impulses were* amplified with a PAR 113 amplifier and displayed on a storage oscilloscope Tektronix 5103N from which they could be photographed They were then fed through an impulse amplitude discriminator It has two variable voltage levels and a time window Only impulses which exceed the lower level but not the upper and which last for less than 1 ms were counted A pulse was given out, which was monitored on the oscilloscope and also added up in a counter *The counter adds up during a predetermined time period* The impulse frequency *obtained* during that period was then presented as a digital signal to a Statos I recorder Varian assoc. In this way the impulse frequency could be plotted as a continuous histogram during the experiment

In the brain 2 ms (long) pulses at different frequencies were used to evoke salivation The current of these pulses ranged from 10 to 150 μA between animals but was always kept constant for a given animal Mechanical stimulation of the tongue was exerted by a *brush of* horsehairs connected to a loudspeaker that was driven by an audio amplifier The amplifier was then driven by a stimulator Grass S48 and S4 stimulators were used

The secretion was measured by absorption of the saliva in a cotton wool bud for 30 or 60 s The increase of weight was determined The accuracy of the method lies within 1 mg After the experiments the brains were fixed and the localization of the electrode in the brain was determined In most cases the brains were fixed and stained with thionin (Ehrlich) *They were* then mounted in paraffin The brains were in most cases cut in transverse section some in sagittal and were examined under the light microscope

## Results

*Location of the stimulation electrodes in the brain*

As mentioned in Methods the localization of the electrode in the CNS was governed by the flow of saliva obtained After the experiments the position of the electrode was determined histologically Fig 1 shows an example of this *The brain was* sagittally sectioned The position of the tip of the electrode is indicated by the circle This spot lies 1 7 mm from the midline In other animals the brains were cut in transverse section Fig 2 summarizes the location of points that produced a salivary






Fig 1 Fig 2

Fig 1 The photograph shows a parasagittal section 1 7 mm from the midline through the rostral portion of the hindbrain and caudal portion of the midbrain The electrode passed through the anterior border of the cerebellum to penetrate the floor of the fourth ventricle with the position of tip indicated by the circle The pons is located in the lower right and the colliculus in the upper right of the photograph

Fig 2 The points of stimulation which produced secretion in 5 rats are schematically illustrated The vertical and horizontal scales are in mm Abbreviations V ventricle V and NMT Nucleus tractus mesencephalic nervi trigemini FLM Fasciculus longitudinalis medialis SBV Tractus spinocerebellaris LC Locus cæruleus VII Nervus facialis VIII Nervus vestibulare

flow of 30 mg/30 s in 5 animals Three points lie dorso lateral and two points lie ventro lateral to the facial genu The general location did not differ in other animals

The possibility that the efferent activity in the chorda tympani proper during the stimulation was the result of direct stimulation of the facial nerve rather than of nuclear structures and consequently that the secretion we studied might have been elicited by a direct stimulation of the chorda tympani proper fibres was checked during the experiments Fig 3 illustrates one method The bottom trace of Fig 3 is composed of 20 superposed records of the efferent activity in the nerve during 20 repeated stimulations in the CNS Pulses having a duration of 0 3 ms and a current of 150 μA were used as stimuli Fig 3 shows that this stimulation gave rise to a burst of efferent impulses If this stimulation had affected the nerve fibres directly a large compound action potential would have been recorded about 1 ms after the



Fig 3 The activity in the chorda tympani proper was recorded as 20 superposed traces after mechanical stimulation of the tongue (upper trace) and CNS stimulation (lower trace) The records show a reflex response to the mechanical stimulation after about 7 ms and discharge of impulses to the CNS stimulation after about 4 ms 2 ms/div

stimulation There is no trace of such a compound action potential after the artifact. The compound action potential might be concealed in the stimulus artifact but because of the short duration of the stimulus (0 3 ms) this seems unlikely

The upper trace of Fig 3 was recorded during 20 repeated mechanical stimulations of the tongue The upper stimulation was applied at 20 Hz 0 3 ms duration for 1 s This trace shows a compound wave of sensory impulses travelling to the CNS in the afferent fibres followed a few ms later by a reflex response in efferent fibres

The inference that the secretion described was not caused by direct stimulation of secretory fibres is more conclusively supported by Fig 4 It shows in the middle trace the efferent activity in the chorda tympani proper displayed as described under Methods The lower trace illustrates schematically the secretion obtained. (The upper trace is a record of the blood pressure monitored as a control of the general status of the animal) Fig 4 consists of two records which were obtained in the same animal before and 13 min after injection with barbiturate 60 mg Mebumal® kg b wt The left record was obtained during the usual anesthesia (see Methods)

The left record shows that during CNS stimulation a secretion was obtained at the same time as the rise in efferent activity After barbiturate a considerable stronger stimulation in the same area of CNS was unable to increase the efferent activity or cause any secretion This shows that the CNS stimulation did not directly affect the secretory fibres to the gland if it had affected them secretion and increase of impulse activity would have been observed after the barbiturate administration also

*Observations which indicate that chorda tympani fibres mediate the effects of the stimulation*

The effect of CNS stimulation on salivary secretion after the chorda tympani proper had been cut was studied in most animals In general no secretion was

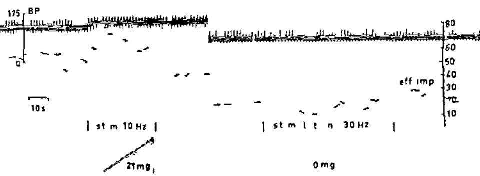



Fig 4 The blood pressure is recorded in the upper trace the impulse activity in the middle The lower trace shows schematically the saliva obtained The left record was obtained during neuroleptic analgesia the right one after barbiturate in the same animal Within the periods indicated by the vertical lines CNS stimulation was applied The figures give the stimulus frequency and secretion obtained The left record shows that the CNS stimulation increased the nerve activity and caused secretion After barbiturate a stronger stimulation gave no increase in nerve activity and no secretion

obtained In one case a flow of 4 mg/min was observed This was about 5 % of the flow when the chorda tympani proper was intact The flow disappeared after ipsilateral sympathectomy below the superior cervical ganglion No secretion seemed to be elicited through the lingual proper nerve in response to the CNS stimulation applied here

It may therefore be concluded that the secretion we obtained from the mandibular salivary gland during the CNS stimulation was the result of orthodromic impulses in the chorda tympani proper nerve originating from neurons in the CNS

*Observations on the relationship between the nerve activity and the secretion*

The main aim of this series of experiment was to record the actual impulse activity which causes the salivary gland to secrete and to describe the relationship between changes in its rate and the secretion caused This relationship was studied in a number of animals Fig 5 shows the results of 27 observations in 5 animals In order to obtain the data shown the efferent activity during 30 s immediately before stimulation was substracted from the efferent activity during the first 30 s of stimulation The increase was expressed as a percentage of the pre stimulation value and the logarithm of these values were plotted on the ordinate while the secretion obtained during the same time period was plotted along the abscissa The correlation coefficient between these two parameters is 0 84 The equation of the regression line of this relationship was calculated and the line drawn in Fig 5 Calculation showed that this semilogarithmic curve better describes this relationship than a linear or power curve fit

When these results had been obtained the chorda tympani proper was cut in most animals and its peripheral part electrically stimulated The secretion we ob-

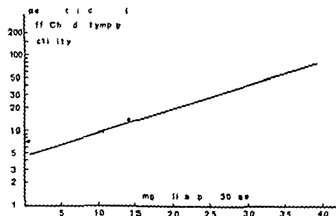



Fig 5 The logarithmic values for the percentage increase of nerve activity during CNS stimulation were plotted against the secretion obtained Each symbol type indicates one animal The correlation coefficient between the two parameters is 0 84

tained in response to the CNS stimulation was not smaller than the secretion we observed during direct stimulation of the nerve up to a frequency of about 20 Hz Much higher stimulation frequencies have not been used because it was not the aim of this investigation to study extreme situations The results obtained show a similar semilogarithmic relationship between stimulus frequency and flow of saliva The correlation coefficient is 0 92

*Other observations of the impulse activity before during and after stimulation*
The left part of Fig 4 shows other characteristics First it should be noted that no secretion was recorded before CNS stimulation although efferent impulses were travelling in the nerve Second though the increased nerve activity apparently caused the secretion the rate of flow of this secretion seemed to be unaffected by the progressive diminution of the nerve activity during the later stage of stimulation Third there seems to be an inverse relationship between the blood pressure and the efferent activity

The right part of Fig 4 shows that injections of barbiturate diminished the spontaneous efferent activity by about 70 % and abolished the effects of CNS stimulation on the efferent activity and salivary secretion It also lowered the systemic blood pressure but not to an extent that this could be expected to be the cause of the ceased secretion

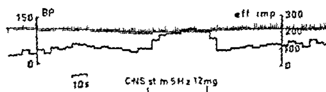



Fig 6 The blood pressure the efferent activity/5 s and stimulation period were recorded as in Fig 4 To make the frequency histogram easier to read the horizontal lines were connected The record indicates no effect on the blood pressure of the stimulation

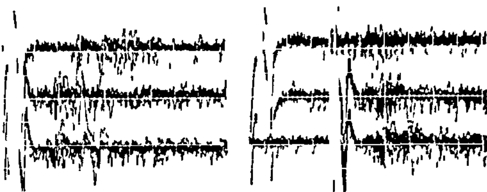


Fig 7 The records were obtained from the central part of the chorda tympani proper in a similar manner to those in Fig 3 The top traces show the reflex response to tongue stimulation The bottom ones show the response to CNS stimulation In the middle records both stimulations were applied but with different delay In the left trace CNS stimulation was applied 0 5 ms after the tongue stimulation in the right, 6 5 ms The left record consists of 5 and the right of 10 superposed sweeps 2 ms/div

*The effects of CNS stimulation on systemic blood pressure*

The left record of Fig 4 shows an effect on blood pressure of CNS stimulation Similar effects were observed in most animals though not in all and not to all stimulation frequencies It was less evident at lower frequencies Fig 6 gives an example of this The record may indicate a small shift in blood pressure but the shift seems not to be related to the stimulation The record shows also that the increase in efferent activity was maintained during the stimulation Finally the period of stimulation was not followed by a period of lowered efferent activity This will be discussed later

*The relationship between the lingual chorda tympani reflex and this study*

Fig 3 shows that reflex activity could be recorded in the efferent chorda tympani nerve in response both to CNS stimulation which gave salivation and to mechanical stimulation of the tongue which is known to cause salivation in the unanesthetized animal It may be surmised that the same type of neurons were active in both cases Therefore experiments were done based on the idea that if the same type of neurons were involved in the reflex response to both stimuli their response to one would block or diminish their ability to respond to the other if the second one occurs within their refractory period Fig 7 presents the result of such an experiment Each trace of Fig 7 is a record from the same chorda tympani proper nerve The nerve was blocked peripherally The left traces are composed of 5 superposed sweeps the right ones of 10 The upper traces were rcorded during mechanical stimulation of the tongue at 10 Hz 1 ms duration The records show a reflex response similar to that in Fig 3 The lower traces show the effect of CNS stimulation with 150 μA 10 Hz and 1 ms duration of the pulses The records show in a

similar way as in Fig 3 efferent impulses as the result of the CNS stimulation In the middle traces both types of stimulation were applied but at different delays. Thus in the left record the CNS stimulation was applied 0 5 ms after the mechanical one The record shows that the CNS stimulation abolished the response to mechanical stimulation In the right record mechanical stimulation was elicited 6 5 ms before the CNS stimulation A decrease and in the right part an abolition of the response to the CNS stimulation can be observed These effects indicate that a considerable portion of the neurons whose activity we recorded in the chorda tympani proper were affected by both types of stimulation

In conclusion, these results show that during the CNS stimulation applied in this study an increase of the efferent activity in the chorda tympani proper nerve is accompanied by a secretion from the mandibular salivary gland This increase in nerve activity was elicited via neurons in the CNS and not caused by direct stimulation of the secretory fibres in the chorda tympani proper The relationship between this increase in efferent activity and the secretion caused is best described if the logarithm of the percentage increase of efferent activity is plotted against the secretion obtained In all animals efferent impulses were recorded which were not accompanied by salivation The efferent activity showed an inverse relationship with the blood pressure changes

## Discussion

The main aim of this series of experiment was to record the impulse activity which causes a salivary gland to secrete and then to describe the relationship between this activity and the secretion caused In order to do this we had to evoke salivation by some means because the secretion normally ceased during anesthesia We chose stimulation in the brain in an area which has been used by other experimentators to evoke salivation The histological sections show that our points of salivation coincide with the general regions described by other workers as the location of the salivary nuclei Thus Wang (1943) reported that points of stimulation which produced salivation in the cat were located in a medial group in the vicinity of the facial genu and a lateral group were located medial and slightly dorsal to the spinal trigeminal nucleus Wang described the lateral group as corresponding to the dorso-lateral portion of the lateral reticular formation Shute and Lewis (1960) delineated the salivary centres in the rat by means of histochemical techniques for cholinesterase and reported the salivary nucleus to be in a position relative to the facial genu ventral to the caudal pole of the nucleus cæruleus which essentially is the same area as the one we have stimulated

The observations after cutting the chorda tympani proper indicate that the *secretion studied here is unlikely to have been elicited* through a neural pathway other than the chorda tympani proper In one animal a small flow was observed which disappeared after sympathectomy This flow was only about 5 % of the flow obtained with the chorda tympani proper nerve intact It can therefore be conclud

ed that the major nerve influence during the CNS stimulation here was mediated through the chorda tympani proper nerve

Further it was essential that the CNS stimulation did not directly affect the nerve fibres to the chorda tympani proper The results of the methods demonstrated in Fig 3 and 4 seem to exclude this If stimulation directly affected the nerve fibres a compound action potential would be observed as was the case in a few experiments Further barbiturate does not abolish the secretion caused by direct stimulation of the fibres in the chorda tympani proper However in this last case it may be suggested that the cessation of salivation was the result of a diminished blood supply because the blood pressure was lower after the barbiturate But this seems improbable since we obtained a normal response to pilocarpine and since other observations show that the glands could secrete at high flow rates at the same or lower blood pressure It is therefore most likely that the CNS stimulation applied here stimulated nuclear structures which then gave rise to the efferent impulse activity we recorded in the nerve and caused the mandibular salivary gland to secrete

It was mentioned that the electrode was not further moved but fixed when a flow of about 30 mg/30 s of saliva was obtained to stimulation After the final preparation electrical stimulation of different frequencies was applied in the CNS If in an animal flows of similar magnitude as the one immediately after the implantation of the electrode could not be obtained it was concluded that some factor like partial blockage of the nerve impulses was operating (Devries 1972) Such an animal was not used in establishing the relationship shown in Fig 5 However straight lines were obtained when these values were plotted in a similar semilogarithmic diagram as the one in Fig 5 but the slope of these lines was steeper If an animal was accepted all values obtained from it were used The number and distribution of data allow the conclusion that the relationship between frequency of secretory impulses and secretion caused is a semilogarithmic one

The finding that an exponential curve fit better describes this relationship than a linear or power curve fit parallels other observations on the relationships between a nervous influence and its effects Thus a similar relationship was observed between the frequency of direct stimulation of the chorda tympani proper or the lingual proper nerves and the secretion obtained (Hellekant and Kasahara 1973 b)

Fig 2 shows an efferent impulse activity of about 50 imp/s before stimulation In spite of this no secretion was observed The possibility that this activity consisted of sensory impulses can be ruled out since section of the nerve peripherally to the recording electrodes did not abolish this activity There seem to be two possible explanations first the impulse activity was below the threshold for the gland or second there is at least one other type of fibre with a different function in the nerve

Observations obtained in t[illegible] rats show that the idea that the nerve activity was below the threshold for the gland is probably incorrect In the first of these animals the initial impulse activity wh[illegible]n no stimulation was applied was about 80/s At the

end of the experiment this activity had increased to 120/s In spite of this 50% increase in efferent activity no secretion of saliva was recorded between stimulations But during stimulation a 5% increase was accompanied by secretion

The second animal showed a cyclic change in efferent activity with a maximum frequency in the middle of the recording period when the rate was 100% higher than at the beginning and the end of this period In spite of this no salivation was observed except during CNS stimulation It can be concluded that if such large changes of efferent activity can occur without giving rise to salivation it is most likely that they emanated from fibres which were not secretory

This points to the second explanation that there are more types of efferent fibres than secretory ones in the chorda tympani proper It is perhaps too early to speculate about the nature of these fibres but during CNS stimulation changes were observed in the efferent activity which were inversely related to the blood pressure changes Thus during stimulation the efferent activity fell and the blood pressure increased while the secretion was maintained After the stimulation the efferent activity was lowered while the blood pressure was elevated The observations in the few cases when salivation could be elicited without an effect on the blood pressure further support this theory Little or no decrease of efferent activity occurred during stimulation and no depression afterwards was recorded

We made a few attempts to distinguish between these possible fibre populations according to their amplitude We were able at the same time to record from fibres which did and did not change their activity during stimulation While there seems to be no consistent difference between the fibres which could be related to the amplitude of their impulses it seems likely that there is at least one further type of efferent fibre in the chorda tympani proper other than the secretory one It is probable that these fibres are related in some way to the vascular system of the animal This corroborates the observations in an earlier study (Hellekant 1972) which suggested that efferent impulse activity in the chorda tympani proper is affected by cardiovascular stimuli

A reflex response in the chorda tympani proper to mechanical stimulation of the tongue was described earlier (Hellekant and Kasahara 1973 a) It was then suggested that this reflex was a part of the basis for the flow of saliva The results of Fig 7 appear to support this suggestion because the reflex response to mechanical stimulation was blocked by CNS stimulation which as previously shown elicited secretory impulses in the chorda tympani proper This indicates that the same neurons were activated in both cases

This discussion suggests that efferent impulses in the chorda tympani proper were evoked by stimulation of nuclear structures in the salivary centre These impulses caused the mandibular salivary gland to secrete The relationship between the increased efferent activity and the secretion can be described by a straight l e in a emilogarithmic diagram There are besides the secretory impulses other efferent impulses in the nerve These impulses are probably related to the vascular control Finally the earlier assumption is supported that the reflex response which can be recorded to mechanical stimulation of the tongue occurs in ecretory fibres

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TABLE II Activities of gastrocnemius muscle enzymes 1–5 days after a single bout of exhaustive swimming with a tail load of 15 g

| Enzyme | Controls | Days after exercise | | |
|---|---|---|---|---|
| | | 1 | 3 | 5 |
| Hexokinase | 11 5 ± 0 1 | 13 3 ± 0 8 | 13 6 ± 0 7[a] | 17 9 ± 0 8 |
| Glycogen synthetase (I form) | 0 75 ± 0 06 | 0 71 ± 0 09 | 0 83 ± 0 07 | — — — |
| Glycogen synthetase (Total) | 5 20 ± 0 26 | 4 58 ± 0 28 | 4 60 ± 0 27 | — — — |
| *Glucose 6-phosphate* dehydrogenase | 2 57 ± 0 08 | 5 00 ± 0 65[d] | 5 53 ± 0 54[d] | 2 38 ± 0 48 |
| 6 Phosphogluconate dehydrogenase | 4 36 ± 0 20 | 6 03 ± 0 35[d] | 6 09 ± 0 80[a] | 4 30 ± 0 45 |
| Glycerolphosphate dehydrogenase | 110 ± 4 | 115 ± 4 | 110 ± 4 | 109 ± 5 |
| Pyruvate kinase | 633 ± 7 | 655 ± 26 | 643 ± 17 | 635 ± 11 |
| Lactate dehydrogenase | 1137 ± 13 | 1307 ± 23[d] | 1193 ± 23[a] | 1123 ± 20 |
| Glutamate dehydrogenase | 2 43 ± 0 07 | 2 75 ± 0 1[?] | 2 41 ± 0 14 | 2 43 ± 0 11 |
| Fumarase | 41 4 ± 0 8 | 46 2 ± 1 5 | 45 9 ± 1 1[c] | 42 3 ± 1 1 |
| Cytochrome oxidase x10[4] | 248 ± 5 | 291 ± 14 | 267 ± 19 | 255 ± 14 |

The activities are exp ssed as μ moles of substrate utilized per min per g dry weight Values are means ± S E of 12 m scles
a = $p < 0\ 05$ b = $p < 0\ 01$ c = $p < 0\ 005$ d = $p < 0\ 001$ (Student s t test)

during 10 min of incubation at 30 C in the presence and absence of glucose 6-phosphate (Thomas Schlender and Larner 1968) Total glycogen synthetase activity (I + D form) was measured in the presence of glucose 6 phosphate and I form activity in its absence

For the determination of the other enzymes studied the muscle extract was prepared by homogenizing frozen powder in 10 vol ice-chilled 150 mM KCl containing 50 mM $KHCO_3$ and 6 mM Na H EDTA (Shonk and Boyer 1964) using an Ultra Turrax disintegrator for 75 s The homogenization was performed at intervals in an ice bath The homogenates were centrifuged at 2 C for 15 min at 30 000 × g and the supernatant was kept on ice and used for all enzyme assays with the exception of cytochrome oxidase which was measured on the crude homogenate The assays were carried out in a Beckman DB-GT recording spectrophotometer with an automatic cuvette positioning attachment at 23 C The reactions were started by addition of an aliquot of the supernatant to the appropriate assay mixture All enzyme activities were expressed as μmoles of substrate utilized per min per g dry weight

Hexokinase and phosphorylase activities were analysed according to Bass *et al* (1969) Cytochrome oxidase was assayed as described by Bostrom and Johansson (1972) The methods described by Shonk and Boyer (1964) with slight modification were used for determination of

TABLE III Activities of cardiac muscle enzymes 1 and 3 days after a single bout of exhaustive swimming with a tail load of 15 g

| Enzyme | Controls | Days after exercise | |
|---|---|---|---|
| | | 1 | 3 |
| Hexokinase | 2 13 ± 0 13 | 2 39 ± 0 10 | 2 34 ± 0 11 |
| Glucose-6-phosphate dehydrogenase | 1 37 ± 0 10 | 2 03 ± 0 28 | 1 79 ± 0 30 |
| 6-Phosphogluconate dehydrogenase | 1 16 ± 0 18 | 1 71 ± 0 31 | 1 34 ± 0 25 |
| Pyruvate kinase | 61 3 ± 6 9 | 75 1 ± 4 3 | 64 ± 3 2 |
| Lactate dehydrogen | 679 ± 43 | 768 ± 20 | 779 ± 15 |
| Fumarase | 64 4 ± 4 0 | 77 7 ± 2 9 | 77 8 ± 3 2 |
| Cytochrome oxidase x10 | 717 ± 60 | 892 ± 47 | 1143 ± 76[d] |

The activities are expressed as μ moles of substrate utilized per min per g dry weight Values are means ± S E of muscles
a = $p < 0\ 05$ b = $p < 0\ 01$ c = $p < 0\ 005$ d = $p < 0\ 001$ (Student s t test)

glucose-6-phosphate dehydrogenase 6-phosphogluconate dehydrogenase glycerolphosphate dehydrogenase pyruvate kinase and lactate dehydrogenase The assay mixtures for the other enzymes were as follows

Phosphofructokinase 50 mM triethanolamine buffer pH 7 6 5 mM $Na_2H_2$ EDTA 10 mM $MgCl_2$ 2 mM cysteine 0 3 mM NADH 3 mM ATP 2 mM fructose 6 phosphate 0 6 U aldolase 0 3 U glycerol phosphate dehydrogenase 0 3 U triosephosphate isomerase

Glutamate dehydrogenase 50 mM triethanolamine buffer pH 7 6, 6 mM $Na_2H$ EDTA 50 mM $NH_4Cl$ 0 1 mM NADH 0 4 mM ADP 10 mM α ketoglutarate

Fumarase 50 mM triethanolamine buffer pH 7 6 5 mM $Na_2H$ EDTA 1 7 mM Na malate

*Estimation of glycogen and RNA*

Glycogen content was analysed in a frozen powder of muscle tissue This was boiled for 20 min in 30 % KOH and glycogen precipitated over night at 4 C in 66 % ethanol with 60 mM $Na_2SO_4$ After 2 ethanol washings the isolated glycogen was determined using an all enzymatic method described by Adolfsson (1973) Glycogen values were expressed as mg glycogen/g dry weight RNA concentration of the muscles were estimated by alkaline hydrolysis (Fleck and Munro 1962) The RNA concentrations are expressed as mg RNA/g dry weight

*Glucose tolerance test*

Exercised and control rats were subjected to intravenous tolerance test according to Nowell and Howland (1966) The glucose infusions were performed at the same time in the morning to exclude diurnal variations The animals were anesthetized with Nembutal (6 mg/100 g b wt i p ) and glucose was rapidly injected in the right femoral vein (0 2 ml of 30 % glucose/100 g b wt ) From the tail vein 0 05 ml of blood was drawn for determination of blood glucose concentration (Hjelm and de Verdier 1963) Blood was drawn before and 10 20 and 30 min after the injection

## Results

Maximal *in vitro* enzyme activities of rat gastrocnemius and cardiac muscle were measured for a number of enzymes 1 3 and 5 days after exhaustive swimming in order to study the duration of enzyme changes (Table II—III) Increased activities in mitochondrial and extramitochondrial enzymes were noted in gastrocnemius and cardiac muscle 1 and 3 days after swimming with a tail load of 15 g No changes in enzyme activities were seen 5 days after exercise The highest relative increase in the activities of the gastrocnemius muscle enzymes was seen in the hexose mono phosphate shunt dehydrogenases The activities of glucose 6 phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were increased 115 % and 40 % respectively 3 days after the exercise In the cardiac muscle the activity of glucose 6-phosphate dehydrogenase was higher after exercise but 6 phosphoglucogenate dehydrogenase was not significantly increased The hexokinase and lactate dehydrogenase activities of the two muscles were increased about 15 % one and three days after exercise The activity of pyruvate kinase was higher in the cardiac muscle 1 and 3 days after exercise but this change was significant ($p < 0.05$) only when the values from 1 and 3 days after exercise were pooled and compared to the control No changes were found in the activities of glycogen synthetase (I and I+D) glycerolphosphate dehydrogenase and pyruvate kinase in gastrocnemius muscle after exercise Of the mitochondrial enzymes the cytochrome oxidase activity of the gastrocnemius muscle was about 15 % higher after exercise while in the heart the increase was about 60 % In both gastrocnemius and cardiac muscle the activity of fumarase was significantly increased 1 and 3 days after exercise In gastrocnemius

TABLE IV Activities of gastrocnemius muscle enzymes 1/2—24 h after a single bout of exhaustive swimming with a tail load of 9 g

| Enzyme | Hours after exercise | |
|---|---|---|
| | 1/2—1 | |
| | Controls | Exercise |
| Phosphorylase | 57 6 ± 1 3 | 57 0 ± 2 6 |
| Hexokinase | 11 0 ± 0 7 | 8 4 ± 0 4 |
| Glucose 6-phosphate dehydrogenase | 2 68 ± 0 16 | 3 14 ± 0 46 |
| 6 Phosphogluconate dehydrogenase | 4 05 ± 0 29 | 4 59 ± 0 45 |
| Phosphofructokinase | 88 6 ± 5 7 | 48 5 ±2 ? |
| Pyruvate kinase | 641 ± 22 | 609 ± 3? |
| Lactate dehydrogenase | 1216 ± 22 | 1?16 ± ?1 |
| Fumarase | 39 3 ± 1 7 | 38 7 ± 1 3 |
| Cytochrome oxidase x10 | 254 ± 21 | 216 ± 14 |

The activities are expressed as µmoles of substrate utilized per min per g dry weight Values are means ± S E of 9 muscles
a = $p < 0.05$ b = $p < 0.01$ c = $p < 0.005$ d = $p < 0.001$ (Student's t test)

muscle the activity of glutamate dehydrogenase was higher 1 day after exercise compared to control muscle

In order to study the onset of changes in enzyme activities measurements were done 1/2—1 8 16 and 24 h after swimming (Table IV—V) It was also investigated whether a single bout of swimming of rats with a tail load of 9 g would give the same enzyme changes as were found with a tail load of 15 g reported above (Table II—III) Since the measurements 8—16 h after the swimming could not be performed in the morning as for the groups studied 1—5 days after swimming data are given for control muscles at all times measured However, as can be seen from these control groups in Table IV—V no diurnal changes were found

In gastrocnemius muscle the hexokinase activity was reduced 1/2—1 h after swimming After 1/2—1 h and 8 h the phosphofructokinase activity was reduced The fumarase activity was significantly increased after 16 h (Table IV) Higher activities of glucose 6 phosphate dehydrogenase 6 phosphogluconate dehydrogenase phosphofructokinase fumarase and cytochrome oxidase were seen in gastrocnemius muscle 24 h after swimming

Reductions in enzyme activities were not seen in cardiac muscle Increased activities of phosphorylase hexokinase and fumarase appeared after 8 h and a rise was also obtained in the activities of phosphofructokinase pyruvate kinase and lactate dehydrogenase 16 and 24 h after exercise (Table V)

Enzyme activities were also studied in gastrocnemius muscle 24 h after swimming with a tail load of 6 g With this tail load the enzyme changes were similar to those found with a tail load of 9 g except that no response was seen in the hexose monophosphate shunt dehydrogenases

As the hexose monophosphate shunt is involved in biosynthetic processes it was of interest to measure the RNA concentration of gastrocnemius and cardiac muscle

| 8 | | 16 | | 24 | |
|---|---|---|---|---|---|
| Controls | Exercise | Controls | Exercise | Con[illegible] | [illegible] |
| 57 2±1 5 | 58 9±2 2 | 57 0±2 6 | 54 2±2 0 | [illegible] | [illegible] |
| 10 6±0 7 | 10 3±0 7 | 11 8±0 7 | 10 7±0 4 | [illegible] | [illegible] |
| 2 54±0 23 | 2 13±0 21 | 2 60±0 12 | 3 00±0 1[illegible] | [illegible] | [illegible] |
| 3 85±0 21 | 3 12±0 29 | 4 09±0 27 | 4 69±0 40 | [illegible] | [illegible] |
| 90 2±6 0 | 53 9±6 5 | 95 1±10 0 | 70 8±12 [illegible] | [illegible] | [illegible] |
| 663±13 | 639±26 | 650±28 | 624±35 | [illegible] | [illegible] |
| 1176±24 | 1203±21 | 1186±49 | 1257±69 | [illegible] | [illegible] |
| 39 8±1 8 | 43 7±1 2 | 39 6±1 4 | 43 7±0 6* | [illegible] | [illegible] |
| 273±14 | 244±15 | 235±11 | 250±8 | 24[illegible] | [illegible] |

A significant increase in the RNA concentration of gastr[illegible] days after exercise and in cardiac muscle 1 day after exer[illegible] Immediately after swimming the RNA concentration of car[illegible] cantly reduced

The glycogen content was reduced about 60 % in the [illegible] 75 % in cardiac muscle 1/2—1 h after swimming with a [illegible] In these muscles the glycogen content increased rapidly [illegible] 16 h and 24 h the glycogen content of the gastrocnemius [illegible] increased above control levels

An [illegible] glucose tolerance test was performed 1 and [illegible] swimming with a tail load of 15 g The day after exercise [illegible] level was reduced (p < 0 05) compared to control rats [illegible] mg/100 ml) A significantly lower (p < 0 05) blood gl[illegible] 186 7±5 6 mg/100 ml) was observed 30 min after gl[illegible] rats The blood glucose values 10 and 90 min after glu[illegible] for exercised and control rat[illegible] 2 days after swimming [illegible] showed values that did not differ from the controls [illegible] glucose tolerance was increased at least one day aft[illegible]

## Discussion

The present study ha[illegible] sh[illegible] a significant rise in a [illegible] extramitochondrial enz[illegible] activities in gastrocnemi[illegible] days after a single period [illegible] swimming to exhaustion [illegible] 5 days after the swimmin[illegible] The enzyme activities [illegible] wer[illegible] [illegible] conditions and may be regarded as relative [illegible] (Pette 1971)

TABLE 1. Activities of cardiac muscle enzymes 1/2—24 h after a single bout of exhaustive swimming with a tail load of 9 g

| Enzyme | Hours after exercise | |
|---|---|---|
| | 1/2—1 | |
| | Controls | Exercise |
| *Phosphorylase* | 13.8 ± 0.7 | 14.7 ± 1.0 |
| Hexokinase | 2.38 ± 0.13 | 2.43 ± 0.12 |
| Glucose 6-phosphate dehydrogenase | 1.59 ± 0.06 | 1.75 ± 0.08 |
| 6-Phosphogluconate dehydrogenase | 1.14 ± 0.03 | 0.99 ± 0.08 |
| Phosphofructokinase | 21.2 ± 1.8 | 24.2 ± 2.5 |
| Pyruvate kinase | 70.7 ± 2.5 | 73.5 ± 2.5 |
| Lactate dehydrogenase | 715 ± 22 | 764 ± 28 |
| Fumarase | 67.1 ± 1.1 | 69.9 ± 2.2 |
| Cytochrome oxidase x10³ | 723 ± 37 | 803 ± 24 |

The activities are expressed as μmoles substrate utilized per min. per g dry weight. Values are means ± S.E. of 9 muscles.
a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.005$, d = $p < 0.001$ (Student's t test).

Regular physical training increases the aerobic capacity of skeletal muscle by increasing the number and size of mitochondria as well as the activity of mitochondrial enzymes (Holloszy 1967, Kraus and Kirsten 1969). The rise in enzyme activities seems to be due to an increase in enzyme proteins as evidenced by a rise in the concentration of cytochrome c and total mitochondrial protein content (Holloszy 1967). A relatively higher increase in the activity of cytochrome oxidase compared to fumarase in this study indicates that the composition of the mitochondria is altered which also has been reported after training (Holloszy 1967). The formation of new oxidative enzymes is probably an adaptive response to the higher demand for ATP by the contractile work.

Of the extramitochondrial enzymes a marked increase was seen in the activities of glucose 6-phosphate dehydrogenase and 6 phosphogluconate dehydrogenase. A

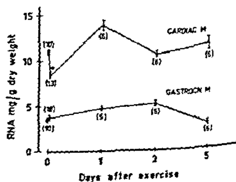



Fig. 1. RNA concentration of rat gastrocnemius and cardiac muscle after a single bout of exhaustive swimming with a tail load of 9 g. The values are expressed as mg RNA/g dry weight and are means ± S.E. Number of rats is given in parentheses and x means significantly different from control, $p < 0.05$. The significance was calculated with Student's t test.

| 8 | | 16 | | 24 | |
|---|---|---|---|---|---|
| Controls | Exercise | Controls | Exercise | Controls | Exercise |
| 13.0±0.7 | 15.6±0.6[a] | 13.4±0.7 | 16.9±0.3[d] | 13.3±0.3 | 21.0±1.2[d] |
| 2.78±0.13 | 3.27±0.09[d] | 2.31±0.09 | 2.85±0.14[b] | 2.43±0.10 | 3.38±0.16[d] |
| 1.53±0.06 | 1.34±0.08 | 1.59±0.07 | 1.44±0.09 | 1.40±0.07 | 1.52±0.11 |
| 1.10±0.03 | 1.05±0.03 | 1.10±0.05 | 1.04±0.03 | 1.06±0.4 | 0.97±0.03 |
| 21.8±1.4 | 23.7±1.4 | 21.1±1.2 | 26.1±1.1[b] | 21.3±1.6 | 30.3±1.7 |
| 66.5±3.2 | 62.7±2.5 | 68.3±2.1 | 69.3±2.8 | 67.6±2.8 | 79.5±3.5 |
| 775 ±15 | 798 ±12 | 767 ±27 | 655 ±50 | 729 ±25 | 886 ±49[a] |
| 69.3±1.1 | 74.3±1.1 | 69.3±2.3 | 76.5±1.7[b] | 67.1±2.1 | 77.6±2.2[c] |
| 726 ±22 | 736 ±34 | 719 ±38 | 707 ±31 | 705 ±40 | 726 ±18 |

similar response was seen in the rat diaphragm with hypertrophy induced by denervation (Turner and Manchester 1970), in isolated rat heart at increasing work load (Nagano and Hochren 1963) and in the gastrocnemius muscle after repeated leg ischemia (Bostrom *et al* 1974). Relatively high activities of hexokinase and the dehydrogenases of the hexose monophosphate shunt are often found concomitantly in various tissues (Novello, Gumaa and McLean 1969, Bostrom and Johansson 1972, Bostrom, Hogberg and Johansson 1973). Increased hexokinase activity might be in

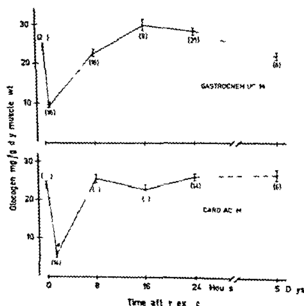



Fig 2 Glycogen content of rat gastrocnemius and cardiac muscle after a single bout of exhaustive swimming with a tail load of 9 g. The values are expressed as mg glycogen/g dry weight and are means ± S.E. Number of rats given in parenthesis and x mean significantly different from controls ($p < 0.05$). The significance was calculated with Student t test

volved in the enhanced hexose monophosphate shunt capacity by increasing glucose 6 phosphate that serves as a substrate for the shunt

One of the major requirements fulfilled by the hexose monophosphate shunt is the production of NADPH for biosynthetic processes and pentoses for nucleotide and nucleic acid synthesis Thus a probable increase in the capacity of the hexose monophosphate shunt is reasonable to find after exercise in view of the increased synthesis of total protein RNA and DNA seen after training and exercise in skeletal and heart muscle (*e g* van Linge 1962 Laugens and Gomez Dumm 1968 Rabinowitz and Zak 1972) An increase in RNA content was also found in the present study both in the gastrocnemius and the cardiac muscle Immediately after the exhaustive swimming the RNA content of the cardiac muscle was decreased which might reflect an increase in catabolic processes Changes of mitochondrial structures (Gollnick and King 1969) and cellular leakage of enzymes (Schmidt and Schmidt 1969) also indicate that degenerative processes may occur at exercise

In the present study a more pronounced response was seen in glycogenolytic and glycolytic enzymes in cardiac muscle compared to gastrocnemius muscle Thus an increase was found in the activities of phosphorylase hexokinase phosphofructokinase pyruvate kinase and lactate dehydrogenase in cardiac muscle 24 h after swimming with a tail load of 9 g while in gastrocnemius muscle an increase only in phosphofructokinase activity was noted Diverging results have been obtained on lactate dehydrogenase activity after exercise (Yampolskaya 1952 Gollnick Struck and Bogyo 1967) Besides the changes in hexokinase and lactate dehydrogenase activities after acute exercise the glycolytic enzyme aldolase has also been reported to increase its activity in rat skeletal muscle after 8 h of treadmill run (Kendrick Jones and Perry 1965) No changes in pyruvate kinase activity of the gastrocnemius muscle was obtained in the present study in agreement with others (Holloszy *et al.* 1971)

The increase in glucose tolerance the day after exercise might reflect increased glucose uptake in muscle Enhanced glycogen synthesis is demonstrated *in vivo* several days after exercise in man and is suggested to be secondary to increased glucose uptake locally in exercised muscle tissue (Bergstrom and Hultman 1966) A significant glycogen overshoot was found in gastrocnemius but not in cardiac muscle in the present study The glycogen synthetase activity showed no increase which is in accordance with studies on human muscle after exercise (Hultman Bergstrom and Roch Norlund 1971) After physical training of guinea pigs (Jeffress Peter and Lamb 1968) and of man (Taylor Thayer and Rao 1972) an increase in total glycogen synthetase activity has however been reported

The intensity of exercise influences the response obtained in enzyme activities. Thus the activities of hexokinase and lactate dehydrogenase were increased in gastrocnemius muscle after swimming with a tail load of 15 g but not with 9 g An increase in the activities of the hexose monophosphate dehydrogenases was found in both gastrocnemius and cardiac muscle with a tail load of 15 g but only in gastrocnemius muscle with a load of 9 g Using a tail load of 6 g there was no response of these enzymes in gastrocnemius muscle

Besides intensity of exercise time of measurement will influence the response obtained in enzyme activities Immediately after exhaustive swimming the activities of hexokinase and phosphofructokinase in the gastrocnemius muscle were significantly reduced A decrease in phosphorylase (Edgerton *et al* 1970) and hexokinase activities (Barnard and Peter 1969 Dohm *et al* 1972) as well as oxidative capacity (Dohm *et al* 1972) has earlier been reported in skeletal muscle immediately after exercise Makarova (1958) has found that directly after exercise there was either an increase or a decrease in the myosin ATPase activity of rat skeletal muscle dependent on the intensity of exercise performed

In contrast to the findings in gastrocnemius muscle there was no decrease in enzyme activities in cardiac muscle immediately after exercise The increase in enzyme activities was also seen earlier in cardiac muscle The activities of phosphorylase hexokinase and fumarase were significantly increased as early as 8 h after swimming Higher activities of phosphofructokinase were found after 16 h and of lactate dehydrogenase and pyruvate kinase after 24 h In gastrocnemius muscle the earliest rise in enzyme activities was seen after 16 h when the activity of fumarase was increased and after 24 h higher activities of cytochrome oxidase phosphofructokinase and the hexose monophosphate shunt dehydrogenases were found

The results illustrate that the different time course of changes in enzyme activities and the intensity of work have to be considered when studying changes in enzyme activities These factors might explain the contradictory results in enzyme adaptation obtained after exercise

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Acta physiol scand 1974 90 555—564

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# Differences in Recruitment Order and Discharge Pattern of Motor Units in the Early and Late Flexion Reflex Components in Man

By

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## Abstract

Grimby L and J Hannerz *Differences in recruitment order and discharge pattern of motor units in the early and late flexion reflex components in man* Acta physiol scand 1974 *90* 555—564

The recruitment order of motor units in the normal and pathological flexion reflex in man was studied by electromyographic techniques The following findings were made (1) the recruitment order is different in the early phasic and in the late tonic flexion component (2) this difference is eliminated so that the recruitment order of the late reflex component is also used in the early reflex component if the motoneurone pool is subliminally facilitated in advance (3) the first motor unit in the early component of the non facilitated pathological flexion reflex tends to discharge a higher frequency than does the first motor unit in the late reflex component It is suggested that short reflex arcs may favour phasic motoneurones while long reflex arcs and long lasting states of facilitation favour tonic motoneurones

The mechanisms regulating the recruitment of individual motoneurones have been extensively studied in animal experiments Henneman Somjen and Carpenter (1965 b) found in reflex studies on decerebrate cat a stereotyped recruitment order determined by the input resistance of the motoneurones in such a way that the small tonic cells are always recruited before the large phasic cells Preston and Whitlock (1963) Sasaki and Tanaka (1964) as well as Clough Kernell and Phillips (1968) however found that the reversed recruitment order may occur on activation from brain centres

In electromyographic recordings single motor unit can be identified by the characteristic shape of their potentials The recruitment order of motoneurones can thus be studied even in man Tonic and phasic motoneurones have different gain and should have different discharge patterns and especially different tendency to attain short discharge intervals Electromyographic recordings may thus give a hint concerning motoneurone type also (see Discussion




Fig 3 General appearance of the reflex in spastic paralyses A strong stimulus and weak pre existing facilitation B weak stimulus but strong pre existing facilitation

The difference in recruitment order between the 2 reflex components can be eliminated The motor unit with the lowest threshold in the late reflex component is usually also the motor unit with the lowest threshold in tonic voluntary activity If the subject facilitates the motoneurone pool subliminally a few seconds before the stimulus this motor unit gets the lowest threshold even in the early reflex component (*cf* Hannerz and Grimby 1973)

Upon habituation the recruitment order in the early reflex component changes in parallel with the inhibition of the late reflex component The recruitment order which was used in the inhibited late reflex component tends to be used in the remaining early reflex component Fig 2 B shows a typical experiment in which the motor unit with the lowest threshold in the late reflex component before habituation is the motor unit with the lowest threshold in the early reflex component after habituation

The discharge pattern of the motor unit with the lowest threshold in the early reflex component in normal man cannot be studied systematically due to the habituation phenomena The motor unit with the lowest threshold in the late reflex component and in tonic voluntary activity discharges mainly at intervals longer than 100 ms in reflex activity but may attain discharge intervals of 40 ms on voluntary driving (*cf* Hannerz 1973)

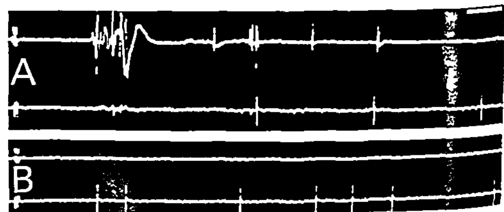
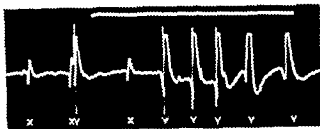



Fig 4 Recruitment order of motor units in the reflex in spastic paralyses Two electrodes inserted at different sites in the muscle (upper and lower traces in each recording) A strong stimulus and weak pre existing facilitation B weak stimulus but strong pre-existing facilitation

Fig 5 Discharge intervals of the first two motor units (marked X and Y respectively) in the early reflex component in spastic paralyses The change in shape of the last Y potentials may be due to a displacement of the recording electrode which cannot be avoided in vigorous twitches




*Paralytic patients* The flexion reflex in patients with total paralyses due to upper motoneurone lesion is similar to that in normal subjects upon unexpected stimuli On moderate stimulus strength a division of the reflex can be made into one early component with a duration of only a few hundred milliseconds and one late component with a duration of several second No significant inhibition of the late reflex component appears upon repeated stimuli The relative strength of the 2 reflex components however varies in a series of reflexes (Fig 3) As shown in a previous study (Grimby 1963) the strength of the early reflex component is directly determined by the stimulus strength while the strength of the late reflex component is more dependant on the pre existing state of facilitation

The recruitment order of motor units is different in the early and in the late reflex components Fig 4 A shows that the motor unit with the lowest threshold in the late reflex component can be inactive in the early reflex component even when this component consists of a considerable number of motor units On the other hand the motor unit with the lowest threshold in the early reflex component can be inactive in the late reflex component also when this component is very strong

The difference in recruitment order between the 2 reflex components can however be eliminated The motor unit with the lowest threshold in the late reflex component is also the motor unit with the lowest threshold in tonic proprioceptive or exteroceptive reflex activity This motor unit gets the lowest threshold even in the early reflex component if the motoneurone pool is subliminally facilitated a few seconds before the stimulus The necessary subliminal facilitation can be built up by passive stretch or vibration of the muscle tendon or by spatial and temporal summation of skin stimuli in facilitating receptive fields *e g* the sole of the foot



Fig 6 Discharge interval of the first motor unit in the late reflex component in spastic paralyses

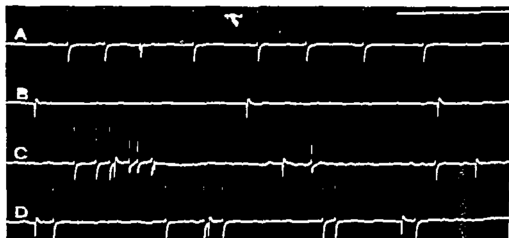



Fig 7 Discharge intervals of two motor units on voluntary driving in subtotal spastic paralyses A, phasically discharging motor unit B tonically discharging motor unit C and D the two units discharging simultaneously

Fig 4 B shows that even very great differences in recruitment order can be eliminated by increasing the pre-existing state of facilitation and the decreasing the stimulus strength

The difference in recruitment order between the two reflex components remains also after repeated stimuli provided that no continuous state of facilitation is built up

In the early component of the non facilitated reflex the motor units with low threshold usually discharge only once but on strong stimuli some of them repeat 2—7 times at intervals of 15—50 ms forming short high frequency bursts Fig 5 shows 2 such motor units the unit marked ↘ discharges at intervals of 25 ms corresponding to a frequency of 40/s the unit marked ↘ is the fastest motor unit observed discharging at intervals of 15 ms corresponding to a frequency of 65/s A motor unit which attain very short discharge intervals in the early reflex component usually has so high threshold in the late reflex component that interference activity from other motor units makes it impossible to study its discharge pattern with the technique used

In the late reflex component the motor units with low threshold repeat at much longer intervals Fig 6 shows the fastest low threshold unit observed in the late reflex component discharging at intervals of 70 ms corresponding to a frequency of 15/s The motor units which have low threshold in the late reflex component discharge at relatively long intervals even in the early reflex component when on strong pre existing facilitation they are active in both components (Fig 4 B)



Fig 8 Recruitment order of motor units in the reflex in parkinsonian akinesia.

The difference in discharge intervals is seen also when the 2 types of motor units are active simultaneously Under favourable recording conditions such as those obtaining in the experiment illustrated in Fig 7 the potentials of one high frequency and one low frequency unit can be studied without interference from other potentials The recordings are from a patient with subtotal spastic paraplegia and the units are activated voluntarily Fig 7 A is a recording of a phasic contraction and shows that the unit which has the lowest threshold in this type of contraction tends to discharge at short intervals Fig 7 B is part of a recording of a tonic contraction and shows that the unit which now has the lowest threshold tends to discharge at long intervals Fig 7 C is a recording of a stronger phasic contraction and Fig 7 D part of a recording of a stronger tonic contraction Fig 7 C and D show that the 2 motor units may have different discharge patterns also when they are active simultaneously Fig 7 B C and D also show that the phasically discharging motor unit has a greater tendency to attain short discharge intervals on increasing level of facilitation indicating that its motoneurone s high gain (see Discussion)

*Akinetic patients* Upon unexpected stimuli the flexion reflex in akinetic parkinsonian patients is similar to that in normal subjects but on repeated stimuli the duration of the parkinsonian reflex is not reduced to the same extent as the normal reflex The well known tendency towards long lasting spontaneous activity in parkinsonism however makes it difficult to decide where the late reflex component ends and spontaneous activity takes over

The recruitment order of motor units is different in the early and in the late flexion reflex components The recruitment order is the same in the late component as in spontaneous activity In the seconds immediately after spontaneous activity has ceased the motor unit recruited first in the spontaneous activity however is also recruited first in the early reflex component The difference in recruitment order between the 2 reflex components is on the other hand not eliminated when the subject is used to the stimulus and expects it

In the early reflex component the first recruited motor unit discharges only once or twice and in the late component only at intervals longer than 100 ms

## Discussion

It was shown in previous studies that the recruitment order of motor units is different in phasic and tonic voluntary activity in normal man (Grimby and Hannerz 1968) as well as in phasic and tonic reflex activity in spinal man (Grimby and Hannerz 1970) It was suggested that the recruitment order of phasic and tonic motoneurones is flexible

It is shown in this study that the motor units are recruited in the phasic order in the early but in the tonic order in the late flexion reflex component and that some motor units recruited first in the early reflex component easily attain high discharge rates in contrast to the motor units recruited first in the late reflex component The large phasic alpha motoneurones have high gain and respond with a

considerable increase in discharge rate to the depolarization that would have produced a much smaller alternation of the firing rate in the small tonic alpha motoneurones (Granit Kernell and Shortess 1963) Our discharge rate findings thus support the hypothesis that phasic motoneurones may be recruited before tonic motoneurones in phasic activity while tonic motoneurones are first recruited in tonic activity Alternative explanations to the difference in discharge rate between the units of the early and late reflex components must however be discussed

Tanji and Kato (1972) have shown that a certain motor unit may discharge at four times higher rate at a phasic contraction than at a tonic contraction It is likely that the motor unit recruited first in the early phasic reflex component discharges closer to its absolute maximum frequency than the motor unit recruited first in the late tonic reflex component However the two motor units have different ability to attain high discharge rate also when they are driven simultaneously (Fig 7) The difference in discharge rate between the motor units of the early and of the late reflex component should partly be due to inherent differences between the motoneurones and their synaptic supply

We have studied the discharge rates mainly in patients with upper motoneurone lesions as habituation phenomena complicate such studies in normal subjects Freund and Wita (1970) have shown that a motor unit may change its discharge rate upon upper motoneurone lesion There are however findings indicating that the original discharge rate of motor unit partially is preserved in pathological states The discharge rate is to a great extent determined by properties of the motoneurone membrane Eccles Eccles and Lundberg 1968 In dogs removal of the motor cortex does not change the discharge rates of motor units in reflex activity (Kosarov et al 1971 Human motor units can be separated into low and high frequency units both in normal and in pathological states on voluntary driving of the anterior tibial muscle the maximum frequencies of different motor units range from 25—65/s Hannerz 1973 the maximum frequencies found for the different motor units in the pathological flexion reflex range from about 15—65/s (Fig 6 and 5

Our results thus indicate that at least some phasic motoneurones have low threshold in the early flexion reflex component but that mainly tonic motoneurones have low threshold in the late reflex component The difference in recruitment order between the two reflex components may be due to differences in synaptic connection of short and long exteroceptive reflex arcs and to feed back effects

The gamma loop participates in the flexion reflex in cat (Eldred and Hagbarth 1954 and probably also in man However only the late reflex component has enough long latency to be linked to the gamma loop It is known from animal experiments that muscle spindle discharge activates tonic motoneurones before phasic motoneurones Henneman et al 1965 Burke 1968) In man blocking of the proprioceptive afferent activity results in considerable changes in the recruitment order of motor units (Grimby and Hannerz 1973) It is probable that the gamma loop contributes to the difference in recruitment order between the early and the late flexion reflex component However deefferentation neither removes

the late flexion reflex component in spinal cat (Andén *et al* 1964) nor reverses the recruitment order in tonic reflexes in decerebrate cat (Henneman *et al* 1965 A) It is not likely that the difference in recruitment order between the early and the late flexion reflex components in man entirely is due to differences in gamma loop participation

If the motoneurone pool is subliminally facilitated in advance the difference in recruitment order between the two reflex components is eliminated so that motor units with presumably tonic motoneurones are recruited first in both components Feed back effects from the early reflex component are thus not necessary for this recruitment order to occur It seems as though the pre existing state of facilitation favours tonic motoneurones while the short reflex arc favours at least some phasic motoneurones

In decerebrate cat the recruitment order of motoneurones is determined by their input resistance in such a way that the small tonic motoneurones are always first recruited (Henneman *et al* 1965 B) This constancy of the recruitment order can be explained by the strong tonic excitatory bias in decerebrate rigidity The flexibility of the recruitment order disappears in man also on strong tonic spasticity (Edstrom Grimby and Hannerz 1973)

In normal subjects habituation changes the recruitment order in the early reflex component in the same way as subliminal facilitation does This indicates that habituation brings about an excitatory bias for tonic motoneurones Habituation however also causes inhibition of those long reflex arcs which favour motoneurones of this type It seems as though upon habituation cerebral centres at the same time programme the motoneurone pool for use of tonic motoneurones and inhibit reflex paths to these units

The habituation phenomena are absent in upper motoneurone lesions causing total paralyses but may also be absent in parkinsonism without clinical signs of pyramidal tract lesion It is likely that the cerebral influence responsible for the habituation phenomena is exterted via extra pyramidal rather than pyramidal structures

The difference in recruitment order between the two reflex components is less in normal man than in paralytic patients It is possible that entirely non habituated reflexes never are studied in normal man It is however also possible that the resting motoneurone pool normally tends to be programmed for use of tonic motoneurones This suggestion is supported by the finding that phasically discharging motor units are earlier recruited in muscles with decreased tone than in normal muscles (Grimby and Hannerz in preparation)

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# Motility of the Urinary Bladder in Cats during Filling at Physiological Rates

## I Intravesical Pressure Patterns Studied by a New Methods of Cystometry

By

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## Abstract

KLEVMARK B *Motility of the urinary bladder in cats during filling at physiological rates I Intravesical pressure patterns studied by a new method of cystometry* Acta physiol scand 1974 90 565—577

Natural filling of the bladder has been studied by a new cystometric method termed Controlled Slow Cystometry (CSC) A suprapubic double-catheter was used for filling from a constant volume infusion pump and for continuous intravesical pressure recording A rate unit and 5 rate models were proposed for the filling procedure The rate unit was defined as the normal hour-diuresis (HD) using 26 ml/kg per day as a normal value The rate models were considered as physiological and relevant to illustrate the normal variations in excretion of urine Maximal physiological increase in urinary excretion was measured to be 15 times the normal Intravesical pressure recording was performed during filling from the pump according to the rate models as well as during normal and increased urinary excretion (5% glucose i v ) A differentiated pressure reaction related to the rate of filling was found Below the rate of 2 4 HD the pressure curve was flat Pressure increase started at individual rates between 2 4—7 HD When the rate fell below this value the pressure stabilized at a higher level (pressure accumulation phenomenon) Accordingly micturition was elicited in the same animal at clearly separated pressure levels depending on the foregoing rates of filling The pressure patterns demonstrated are considered as basic motility reactions present during natural filling

Natural filling of the bladder is a relatively slow process although there are large variations in its rate An increase in urinary excretion of 15 times the normal rate is within the physiological variation in cat and man (Blegen 1940 p 11 O Connor 1962 p 123 Ganong 1971 p 518 see Results)

Due to osmoregulatory mechanisms changes in the rate of urinary excretion *e g* after water intake are not instantaneous but take place over a period of minutes However during emotional stress and muscular exercise rapid reductions in the rate of urinary excretion can occur (Wilson *et al* 1925 Rydin and Verney 1938)

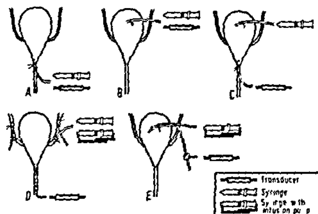



Fig 1 Schematic presentation of technical surgical development of Controlled Slow Cystometry (CSC) *A* and *B* show a commonly used approach *C* *D* and *E* demonstrate the development of CSC

At a normal rate of excretion urine is collected in the renal pelvis and the upper part of the ureter Portions of urine are intermittently ejected into the bladder usually with a frequency of 1—5 per min (Kiil 1957 p 54) The transfer of urine to the bladder remains intermittent within the range of physiological rates (Kiil 1964 p 103)

Studies on bladder physiology have concentrated on mechanisms of nervous control often influenced by classic neurological concepts (Ruch 1960) Filling procedures and myogenic properties have attracted less attention While the main features in excretion of urine and in ureter motility long ago have been explained natural filling of the bladder has not been systematically explored What is the normal bladder motility during the collecting phase? *Do differentiated pressure responses to filling exist within the variations of physiological rates?* This question cannot be answered by applying the common cystometric procedures because the infusion technique whether continuous or intermittent manual or from a bottle is not sufficiently exact Neither is the excretory cystometry (Comarr 1957 b) a suitable method since stimulation of the kidneys to excrete urine at various physiological rates would be too haphazard to yield consistently reliable quantitative results

In the present study a new cystometric procedure has been introduced where the natural filling is augmented by infusion into the bladder from a syringe attached to a multispeed constant volume infusion pump Thus controlled slow filling can be obtained and the main types of physiological variation in rate of bladder filling can be mimicked This method has been termed Controlled Slow Cystometry" (CSC) The pump is the key device for making this filling method reliable and reproducible

The results obtained in addition provide information relating micturition threshold to intravesical pressure and bladder volume

## Methods

*Technical surgical development of the method*

A commonly used approach for recording the pressure volume curve is through a tubing inserted at the bladder neck or vertex as shown in Fig 1 A and B respectively Pressure recording and fluid injection take place through the same catheter The approach shown in B is used when natural emptying is desirable a technique introduced for clinical use (Sand e

Bryndorf and Gertz 1959) and further adopted for experimental purposes (Gjone and Setek ...
1963)

The techniques employed in the present study are presented in Fig 1 C D and E Fig 1 C shows manual injection through a tube introduced at the vertex while pressure is recorded continuously through a catheter inserted at the bladder neck This technique was used only in a few preliminary experiments In D the bladder itself is left unaffected by operative procedures The urine is diverted from the proximal ends of the ureters The catheter for filling is placed in the urethra through a longitudinal slit which is sutured with fine interrupted stitches to avoid a circular ligature at the bladder neck Procedure D was mainly used in the experiments with filling from the pump at lower physiological rate levels

The final arrangement is shown in Fig 1 E A stiff walled double-catheter was constructed from two single catheters with bores of 1 5 mm and 0.5 mm respectively which were glued together from the apex to 2 cm centrally The thicker one for pressure recording reached 2 mm further into the bladder cavity The catheter was inserted into the vertex of the bladder and the stab wound in the bladder was closed around the catheter with purse string suture using an atraumatic type of needle which was placed intramurally This was most easily performed on the contracted thick wall of the empty bladder The suture was placed a few mm from the wound edge using a cuff which when secured around the catheter prevented any leakage The double-catheter could slide freely through the closed abdominal wall Intravesical pressure was recorded through a Statham pressure transducer connected to a Grass Model 7 Polygraph which gave pressure recording with an accuracy of 0 5 cm $H_2O$ A Harvard multispeed constant volume infusion pump was used for filling the bladder This cystometric procedure provides a continuous intravesical pressure recording both during natural filling and pump infusion.

*Definitions and filling procedures*

The new method necessitates the introduction of a defined rate unit for the filling procedure Furthermore the present and subsequent studies necessitate definitions of certain rate models

*The rate unit (HD)* It was found practical to define a rate unit as the calculated normal hour-diuresis (HD) The daily volume in the adult cat ranges from 23 to 29 ml per kg (Spector 1956 p 341) In the present study therefore 26 ml/kg per day has been chosen as a normal value These figures correspond well to data obtained in 10 control expts on anesthetized animals Supplementary infusion was supplied from the pump giving the total number of HD units which were required for the purpose of each expt. The bladder volume was measured at the end of each expt. and the excretion of urine calculated

*The rate models* The following types of bladder filling have been proposed as physiological and relevant to illustrate the normal variations in excretion of urine Continuous filling at a low rate continuous and intermittent high rate filling increasing and decreasing filling rate

*Filling procedures* The intravesical pressure curve has been investigated on anesthetized and non anesthetized animals during

(1) Filling of the bladder from a constant volume infusion pump
  (a) at lower physiological rate levels (approx lower 1/3)
  (b) at upper physiological rate levels (approx upper 2/3
(2) Filling of the bladder by the cats *normal* excretion of urine
(3) Filling of the bladder before during and after stimulation of urinary excretion by i v fluid (5 % glucose administration
(4) Filling of the bladder from a constant volume infusion pump through ureteral catheters compared with filling through a transmural vesical catheter Only performed on anesthetized animals

In addition a few pressure volume curves were obtained using intermittent rapid filling

*Animals and operational procedures*

The results reported in this study are based on data obtained from expts on 51 adult cats of both sexes weighing 1 8—5 3 kg

The animals were anesthetized with pentobarbital sodium (Nembutal® Abbott) i p in doses of 30 mg/kg b w. Small supplementary doses were given i v as required In expts on non anesthetized cats the operations were performed under ether anesthesia. The cystometry was done in the restrained cats at least one hour after the discontinuation of the ether The animals were kept in the supine position laying on a heated pad Rectal temperature was maintained at about 3 C. The fluid from the infusion pump was saline at 37 C The blood pressure was measured with Statham pressure transducer by means of tubings inserted into the femoral artery Thoracic respiratory movements were recorded on the polygraph by a Grass ... pneumatic pressure transducer type PT 5A The anesthetized animal were breathing spontaneously through a tracheostoma To maintain a good general condition in a few prolonged experiments 5 glucose and 0 9 saline were administered i v in moderate doses

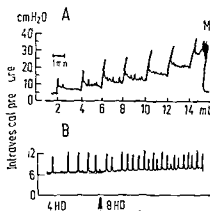



Fig 2 (*A*) cat 2 4 kg Cystometry using intermittent rapid filling with injection of 2 ml saline every 2nd min Immediate rise in pressure was followed by incomplete pressure accommodation Micturition at (M) (*B*) cat 2 7 kg Cystometry at physiological rates A small increase in filling rate (4 HD or 0 ? ml/min) at arrow induced a gradual pressure increase

## Results

The results obtained demonstrate the existence of a differentiated pressure reaction of the urinary bladder during natural filling and pump infusions at physiological rates *The various intravesical pressure patterns were found to be related to the rate of filling* and were in their principal features independent of the actual bladder volume Micturition was elicited in the same cat at clearly separated pressure levels depending on the foregoing rates of filling

*A Effects caused by the experimental procedure*

The use of an unphysiologically rapid and gross infusion gave an immediate pressure rise which was followed by a fall in pressure (accommodation) (Fig 2 A) A small increase in physiological rate resulted in a gradual increase in pressure (Fig 2 B) After quick infusions accommodation did not exceed 1/2 h in the majority of cases This had to be taken into account when slow filling was initiated after a basic volume was obtained by such instantaneous infusion

The bladder has the characteristics of both single unit and multi unit smooth muscle (Prosser 1967 Hald 1969 p 15) Thus motility is affected by physical factors such as operative manipulations and temperature changes Edvardsen (1968 a) demonstrated that excitation elicited by laparotomy was most pronounced immediately after closure of the abdominal incision This was confirmed in the present study Postoperative excitation did not last more than one half hour

Respiratory movements sometimes influenced the intravesical pressure recording The degree and type of respiratory influence depended upon the depth of anesthesia whether the glottis was closed or not and upon the position of the animal In the supine position with sufficient depth of anesthesia to cause a mainly abdominal type of respiration the intravesical pressure of a fairly well filled bladder could fall a maximum of 0 5 cm H O as the inspiratory movement elevated the abdominal wall from the bladder During the expiratory movement the opposite change took place During lighter degrees of anesthesia in which a costal type of respiration p e

dominated it was found that the inspiratory movements could increase and the expiratory decrease the intravesical pressure by a maximum of 0 5 cm $H_2O$

With the glottis closed or very narrowed the non anesthetized animal was able to increase the intra abdominal pressure considerably during muscular straining Usually the corresponding increase in intravesical pressure (15—20 cm $H_2O$) was of very short duration and presented no diagnostic difficulties with respect to rhythmic or micturition contractions of the detrusor muscle

As a consequence of the observed possibilities of nonspecific side-effects the expts were all done with the animal in the supine position and preferably under light anesthesia Postoperatively at least 40 min were allowed for intra abdominal adaptation of the bladder before recordings were taken

*B Intravesical pressure patterns during filling at physiological rates*

(1) *Filling of the bladder from the infusion pump*

(a) *At lower physiological rate levels* the infusion pump was used in 4 expts where the rates of filling varied between 1 7 and 2 4 HD The filling lasted from 3 h to 6 h and 20 min (average 5 h) and started from initial volumes of less than 5 ml A natural micturition contraction occurred in all animals There was no increase in the intravesical pressure during the collecting phase (Fig 3 A) On the basis of these expts and those reported elsewhere in this study the threshold of pressure increase ranged from 2 4 to 7 HD

(b) *At upper physiological rate levels* the intravesical pressure patterns were recorded in 22 cats during the types of physiological bladder filling described above i.e. continuous and intermittent high rate filling increasing and decreasing filling rate

Switching from a filling rate of less than 2 4 HD to one of 8 HD immediately increased the intravesical pressure which at the same rate was apparently linear until micturition occurred (Fig 3 B) If this high filling rate (8 HD) was suddenly reduced below 2 4 HD (Fig 3 C) the intravesical pressure fell initially but stabilized at a higher level This will be termed the pressure accumulation phenomenon If the filling rate was increased or decreased by markedly separated steps for example 7 5 and 15 HD the intravesical pressure revealed stepwise changes as well (Fig 3 D and E)

(2) *Filling of the bladder by the cats normal excretion of urine*

The intravesical pressure was recorded in 7 anesthetized cats with an initial vol[illegible] of 5 ml or less The smallest volume which kept the catheter lumen open wa[illegible]d Micturition contraction ocurred within 2 5 to 15 h (average 7 5 h) In five [illegible]al spontaneous micturition occurred in the other two it was provoked by an [illegible] injection of 0 10—0 18 mg carbacholine after 8 5 and 15 h respectively whil[illegible] the animal were still in a good general condition

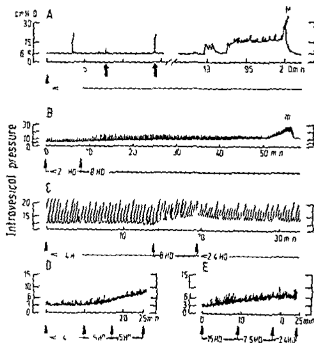


Fig 3 Intravesical pressure patterns at lower and upper physiological rate levels in cats (A) Continuous filling at a low rate (1 7 HD) Note the constant pressure during the collecting phase until active contraction preceded micturition (M) Arrows indicate pressure rise due to straining with narrowed or closed glottis Non anesthetized cat (B) Continuous filling at a high rate (8HD) caused a linear rise in pressure ending in the actual experiment in an abortive micturition contraction (m) (C) At intermittent filling at a high rate (8 HD) the linear rise in pressure was succeeded by a fall but stabilized at a higher level (pressure accumulation phenomenon) (D) Increase in filling rate (< 2 4 HD → 7 5 HD → 15 HD) caused increasing steepness of the pressure curve (E) Decrease in rate (15 HD → 7 5 HD → < 2 4 HD) caused the opposite changes B—E Pentobarbital anesthesia

During the collecting phase the intravesical pressure remained constant in all animals until the moment of active detrusor contraction leading to micturition (Fig 4) In this series of expts the bladder volumes were measured in 3 cats to 19 23 and 39 ml The estimated average urinary excretion was approximately 1 HD

*(3) Filling of the bladder before during and after stimulation of urinary excretion by intravenous fluid administration*

In order to see whether the results obtained with the CSC method could be considered as pressure models reflecting principal features of bladder motility the intra

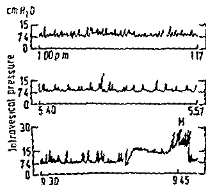


Fig 4 Cat 2 1 kg Intravesical pressure curve obtained during natural filling Micturition occurred after 8 h 45 min at a volume of 19 ml Tracings show 3 representative intervals Micturition (M) was preceded by a continuous detrusor contraction of 4 min duration

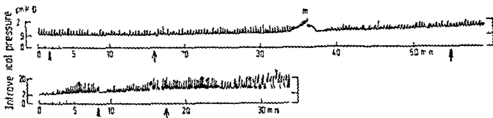



Fig 5 Intravesical pressure following i v stimulation (5 % glucose) of urinary excretion Upper record cat 5 3 kg Start of i v infusion at arrow to the left A gradual rise in pressure after 16 min (second arrow) Abortive micturition contraction without leakage at (m) I v infusion turned off at arrow to the right. Lower record cat 2 9 kg Discontinuation of i v infusion at left arrow The rise in pressure continued for 9 min (right arrow) thereafter the pressure stabilized at a higher level

vesical pressure patterns were recorded before during and after i v stimulation of urinary excretion in 5 animals

Glucose (5 %) was administered i v at a rate of 20—25 per cent of estimated blood volume per hour In preliminary expts this was found to give a maximal increase in urinary excretion of 15 times the normal rate of filling without any complications of hydration After 10—20 min an increase in the intravesical pressure ensued (Fig 5 upper record) The rise in pressure was even and smooth with a slight downward convexity When the i v infusion was discontinued the pressure increased for another 9—20 min and thereafter stabilized at a higher level but without any initial fall (Fig 5 lower record) The decrease in pressure rise was even and smooth with a tendency of upward convexity Measurement of the bladder volumes corresponded to an average filling rate of 3 6—6 HD which meant that maximum rate of excretion had been considerably higher

Thus experiments with natural filling confirm the physiological relevance of 3 of the 5 pressure models obtained by CSC (Fig 3 A D and E) Experimentally induced continuous high rate of urinary excretion is very difficult to obtain as is rapid reduction However CSC through the ureter can simulate such filling reliably Consequently the two remaining pressure models (Fig 3 B and C) are physiologically relevant Thus the intravesical pressure patterns present during natural filling are represented by Fig 3 B and C Fig 4 and Fig 5 A schematic presentation of all patterns is given in Fig 6

(4) *Filling of the bladder from the infusion pump through ureteral catheters compared with filling through a transmural vesical catheter*

The significance of the two factors distinguishing CSC from natural filling i e the intermittent ureteral emptying and the more gradual changes in rate of urinary excretion was evaluated in expts

Both ureters were catheterized through laparotomy and the bladder filled via the infusion pump as in Fig 1 D Thus normal ureteral emptying was not interfered

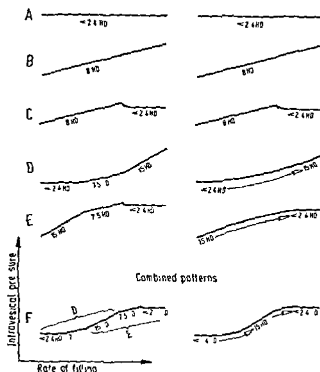



Fig 6 Schematic presentation showing the relationship between intravesical pressure patterns obtained during Controlled Slow Cystometry (CSC) according to rate models and during natural filling (*A*) No increase in pressure at rates below 2 4 HD (*B*) Continuous high rates gave a linear rise in pressure (*C*) When the high rate was rapidly reduced the pressure initially fell but stabilized at a higher level (*A*) (*B*) and (*C*) were identical for CSC and natural filling (*D*) and (*E*) Increasing and decreasing filling rate resulted in stepwise pressure changes during CSC while during natural filling smooth pressure curves ensued

with but changes in rate of filling were artificial When changing from 6 4 HD to 12 8 HD only slight changes in linear rise of pressure occurred When filling through a transmural vesical catheter the change in linear rise in pressure was significantly greater indicating a less adaptive effect of the bladder when ureteral emptying was no longer present

*C Experiments using non anesthetized animals and different operative approaches*
The possible influence of anesthesia was controlled by expts performed on 5 awake cats All surgery was performed under initial ether anesthesia Animals were in the supine position in a comfortable cage and the cystometric measurements started 1 h after termination of the anesthesia

All the principal features of intravesical pressure reactions demonstrated in the preceding expts were reproduced in the non anesthetized animals The basic finding that no rise in the intravesical pressure could be observed at normal filling rate was demonstrated in all 5 animals

In 6 animals several variations of catheter insertion were tried to test the possible influence of the operative procedures *per se* In addition to the approaches described in Fig 1 D and E cystometric measurements were performed through a trans

urethral catheter leaving both bladder and ureters untouched as well as by filling through one ureter and recording through the other leaving both bladder and urethra unoperated No influence of the operative procedures on the intravesical pressure patterns during filling could be found

## Discussion

The cystometric method described above (CSC) allows a control of slow filling rates and hence the natural filling of the bladder can be studied Various physiological filling rates evoked different pressure responses and micturition was elicited in the same animal at various intravesical pressure levels

*Methodological considerations*

The cystometric procedure using intermittent rapid filling renders impossible the observation of differentiated pressure responses as those obtained at physiological rates The former method has not been shown to have a theoretical basis in natural filling It has been conventionally applied in bladder physiology and clinical work (for a survey see Gjertsen 1960 Emmett and Greene 1964 Hald 1969, p 25) The procedure is rather rough and unphysiological as will be seen from the following A cat weighing 2 8 kg has an estimated normal urinary excretion of 0 05 ml per min (Spector 1956 p 341) In cats cystometry is frequently performed by rapid injections of 2—4 ml of saline every second min (Fig 2) Thus the stretch stimulus at the moment of injection is amplified with a magnitude of several hundred times the normal However the administration of stretch stimulus over a period of several injections is much less due to the 2 min intervals Thus in the quoted example the rate of administration of volume is 20—40 times the normal

These 2 factors the rate and the frequency of injections have a potentiating effect on the steepness of the pressure volume curve The rate of injection will determine the degree of acute pressure rise This rise is followed by a period of pressure fall which is interrupted, usually every second minute with the result that accommodation remains incomplete and there is an artificial pressure accumulation throughout the cystometry The steepness of the curve will further be influenced both by the inaccurate infusion technique and by the physical state of the bladder wall (Ruch 1960 Klevmark unpubl) The slope of the pressure volume curve thus obtained is used in the clinic and in experimental research as the operational definition of the tone in the bladder wall (Ruch 1960 Plum 1962 p 155) However considerable scepticism has been emphasized concerning the value of the slope of the pressure volume curve (Watkins 1934 Munro 1936 Comarr 1957 b)

This filling procedure has been critizised as unphysiological and modification towards a more even and slower filling have been developed (Munro 1936 McLellan 1939 Bors and Comarr 1971 p 149 a.o ) Boyarsky and Ruskin (1970 p 124) recommend cystometry with filling at a near maximal physiological rate (10—30 ml per min in man) Recording of intravesical pressure during urinary excretion

stimulated by water loading (excretory cystometry) has been used in clinical studies on neurogenic bladder disorders, e g to evaluate capacity problems (Comarr 1957 b, Tsuji Kuroda and Nakajima 1960) However filling at physiological rates has not been systematically applied in experimental bladder physiology

Pressure curves obtained in the present study during normal excretion of urine are flat and any increase in pressure resulting from an increase in rate is gradual and not followed by a fall in pressure except on occasions with a rapid reduction in high rate urinary excretion The pressure rise obtained by rapid injections demonstrates resistance to sudden stretch which is explained by viscous properties of the bladder wall (Remington and Alexander 1955) By filling within physiological rates such injection artifacts are avoided showing that the bladder wall is calibrated for its normal stretch stimulus Thus the CSC method satisfies this requirement Furthermore the rate unit is defined according to normal excretion of urine and the infusion pump provides reliable filling at low rates These factors and the proposed rate models permit a systematic study of intravesical pressure patterns The results thus obtained facilitate the interpretation of pressure curves acquired during natural filling For the latter purpose the filling procedure sometimes has to be rather time consuming In that ca e it is said that the animal may deteriorate and this limits the number of determinations (Ruch 1960) This objection can only be applied to filling at a normal rate filling at upper physiological rate levels as made possible by CSC is a practical procedure both experimentally and clinically

Natural filling differs from the CSC method in two respects (i) natural filling is intermittent and ii) the changes in rate of urinary excretion are more gradual However these factors have no principal influence on the pressure patterns obtained but improve the intravesical adaptation

The surgical procedure applied in the CSC method is relatively limited and affects only a very small part of the bladder wall Thus the inherent motility of the ureters and the bladder neck with the urethra is unaffected During micturition the opening of the bladder neck can take place undisturbed The bladder volume can be measured at the end of the experiment and compared with the HD value used and thus the average urinary addition can be calculated In the present study the values are usually below 1 HD and do not exceed 2 HD Variations in the rate of urinary excretion *during* a procedure cannot be recorded However with increasing numbers of HD units delivered from the infusion pump the side effects of possible haphazard changes are proportionally diminished The supine position and short insertion of the catheter with fixation to the top of the bladder reduce the influence of changes in hydrostatic pressure during filling

*Bladder reaction to natural filling*

The rate dependent intravesical pressure patterns are concerning their principal features constant and can be considered as basic motility reactions They have not been recognized previously probably due to the lack of a method incorporating the concept of natural filling variations Excretory cystometry used in clinical work

(Comarr 1957, Tsuji *et al* 1960) gives recordings resembling Fig 3 B in the present study However the significance of these recordings has not been further evaluated Discussions of natural filling are generally based on pressure volume curves obtained by a quick filling procedure Consequently descriptions are vague expressing a great increase in volume with only little increase in pressure (Langley and Whiteside 1951 Nesbit and Lapides 1959 Monnier 1968 p 456 Hald 1969 p 18 a o )

The rising pressure curve obtained by i v stimulation of urinary excretion exhibits a smooth form demonstrating intravesical adaptation The mechanisms of adaptation are related to properties of the bladder wall since it is present in the extramurally denervated bladder (Klevmark unpubl ) Smooth muscles show rate dependent tension that may be interpreted in terms of viscosity which tends to damp rapid changes of length (Winton 1930) Applied to the bladder this means that when the filling rate changes the viscous mechanisms may damp the change in length giving the pressure curve its smooth form According to the pressure patterns demonstrated the various conceivable combinations of rate will consistently result in smooth generally rising pressure curves

Why pressure increase in the present study begins at such varied individual rates as 2 4 to 7 HD is not easily understood It most probably is related to viscous properties and hence dependent on the physical state of the bladder wall It should be emphasized that as a rule the degree of pressure rise evoked by a certain physiological rate is only the same when filling to equal volumes in the same cat

*Intravesical pressure patterns and micturition threshold*

The rate of urinary excretion has been mentioned as a possible factor affecting micturition threshold but the significance of this factor has not been further investigated (Bell Davidson and Scarborough 1961 p 634 Root 1968 p 1833 Starling and Evans 1968 p 737) Intravesical pressure at the moment of activation of the micturition reflex has been referred to with uncertainty The expression frequently encountered is that micturition takes place at a certain intravesical pressure (Starling and Evans 1968 p 737 Root 1968 p 1833 a o ) During intermittent rapid filling micturition has a tendency to take place at approximately the same intravesical pressure level in the same cat The intravesical pressure has therefore been considered as the main peripheral factor which elicits the micturition reflex (Edvardsen 1968 b) Finally Iggo (1955) has discussed receptor sensitivity to stretch or intramural tension but without giving precise data about the physical situation related to micturition thresholds On the whole the literature reveals a lack of data and reasoned explanations of the simple situation of intravesical pressure volume relationship when micturition is elicited

The present results show that micturition is elicited at clearly different intravesical pressures During continuous filling at a low rate the intravesical pressure will stay low and constant while the change in volume will influence the threshold of micturition (Fig 3 A and Fig 4) At a continuous filling at a high rate the pressure and the volume increase simultaneously when micturition is elicited

(Fig 3 B) At an intermittent high filling rate the intravesical pressure stabilizes at a higher level when the rate returns to a low value (Fig 3 C and Fig 5) This pressure accumulation phenomenon reflects properties of the bladder wall since it is present in the extramurally denervated bladder (Klevmark unpubl) It reveals an important aspect of bladder motility during the collecting phase Depending on the rate of urinary input, the bladder can build up and maintain higher levels of intravesical pressure In other words, the pressure level at which the micturition reflex is activated is determined by the foregoing rates of urinary excretion

## Addendum

The rate dependent variations of pressure in the normal filling pattern show that micturition is elicited in the same animal at different combinations of pressure and volume Pressure and volume act together and this can be expressed by Laplace's equation $T = \frac{P\ r}{2}$) for intramural tension in a sphere with an elastic wall,

$$T\ 2\pi r = P\ \pi r^2$$

$$T = \frac{P\ r}{2} = \frac{0.31\ P\ \sqrt[3]{V}}{2}$$

$$(V = 4/3\,\pi r^3\ D\ v\ s\ r = \sqrt[3]{(3/4\,\pi)\ V} = 0.31\ \sqrt[3]{V})$$

where T is intramural tension acting along the circumference ($2\pi r$) equilibrating intravesical pressure P acting on the cross section ($\pi r^2$)

The micturition reflex in the same animal is probably activated at a certain value of intramural tension (Iggo 1955) an assumption supported by the present results If so the equation expresses the fact that the bladder can achieve large volumes at low pressures which according to the present results means when the rate of urinary excretion is low Furthermore increases in rate above a certain value will increase the pressure and thus lower the volume threshold for micturition Tension is proportional to the pressure and to the cube root of the volume Thus a change in pressure has a far greater ability to change the tension Such a mechanism might be related to the fact that reflex micturition often occurs at different volumes

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Acta physiol scand 1974 90 578—582

From the Department of Physiology University of Goteborg Sweden

# The Effects of Intestinal Vasodilator Mechanisms on the Rate of $^{85}$Kr Absorption in the Cat

By

BJORN BIBER

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## Abstract

BIBER B *The effects of intestinal vasodilator mechanisms on the rate of $^{85}$Kr absorption in the cat* Acta physiol scand 1974 *90* 578—582

The absorption rate of $^{85}$Kr from the small intestine of the cat was estimated during intestinal vasodilatation induced by transmural electrical field stimulation or by local i.a. infusions of cholecystokinin and secretin As blood flow increased a considerably augmented rate of absorption was observed the absorption rate being increased in excess of that observed during corresponding hyperemia induced by isopropylnoradrenaline An increased fraction of the intramural blood flow became fully equilibrated with luminal contents during the vasodilatory procedures

Recent experimental observations seem to indicate that the functional hyperemia in the gut after a meal is partly of hormonal origin due to a release of cholecystokinin and secretin partly caused by a local vasodilator reflex mechanism elicited via mucosal mechanical (and perhaps also chemical) stimulation by the luminal contents The hemodynamic adjustments in the intestine induced by these various mechanisms have been analysed with respect to the effects in the series-coupled vascular sections (Biber Fara and Lundgren 1973 b) The results suggest that the dilatation of the resistance vessels is accompanied by a relaxation of precapillary sphincters increasing the functional capillary surface area available for transcapillary exchange probably also the absorption capillary surface area

The present study was undertaken to investigate the effects of the above mentioned local flow adjustments in the small intestine with respect to their influence on absorptive events For this purpose model experiments were performed studying the absorption of an inert radioactive gas $^{85}$Kr with a recently developed technique (Biber Lundgren and Svanvik 1973 c) Such a study would also provide indirect evidence whether any local redistribution of intestinal blood flow towards the absorptive mucosal layers takes place during the functional hyperemia

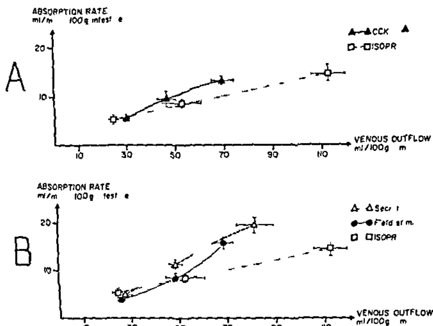



Fig 1 Cumulated data on the correlation between the rate of $^{85}$Kr absorption and total intestinal blood flow as varied by transmural electrical field stimulation or by local i a in fusions of cholecystokinin (CCK) and secretin Lines drawn by inspection Bars indicate ± S E For comparison is also shown the corresponding relationship as seen during a hyperemia induced by isopropylnoradrenaline (Biber Lundgren and Svanvik 1973 c)

## Methods

*A Operative procedures and blood flow recordings* The experiments were performed on 12 cats deprived of food at least 24 h Anesthesia was induced with ether and maintained with chloralose (*40—60 mg/kg b w* )

The operative procedures were similar to those of earlier studies (for details see *e g* Biber *et al* 1971) A proximal jejunal segment and its lymph nodes weighing together 15—35 g was isolated and left *in situ* while the remaining intestine was removed Venous outflow was recorded by means of an optical drop recorder operating an ordinate write All splanchnic nerves and nerves running along the superior mesenteric artery were sectioned Arterial blood pressure was monitored from a femoral artery with a Statham pressure tran ducer (P23AC) all recordings being monitored on a Grass polygraph Local i a. infusions were given *via* a catheter in a small branch of the superior mesenteric artery By occluding temporarily the mesenteric vessels it was possible to measure separately the blood flow in lymph nodes and mesentery and thus to calculate the intestinal blood flow proper

*B Isotope technique and luminal perfusion system* For theoretical considerations and detailed technical description the reader is referred to Biber Lundgren and Svanvik (1973 c) Briefly the intestinal segment was cannulated with glass tubes and perfused with body warm saline in a closed ci uit system about 10 mCi of $^{85}$Kr being dissolved in the perf sate Samples of this perfusion fluid and of the venous effluent could be taken intermittently for measurement of γ radioactivity in a well type scintillation detector The radioactivity of the intestinal venous blood ($C_b$) was also monitored continuously in a second well-type scintillation detector The mesentery was covered by Mylar® (Du Pont) to eliminate diffusion of $^{85}$Kr from blood and tissue to air

As total venous outflow ($Q_{tot}$) from the intestinal segment was measured continuously the absorption rate could be expressed as $C_b \times Q_{tot}$ and compared to the concentration of $^{85}$Kr in the lumen (Cl) The amount of blood (Q ) expressed in ml/min × 100 g that was fully equilibrated with the luminal contents could also be calculated as $Q = C_b \times Q_{tot} \times s / Cl$ where s represents the water blood partition coefficient for $^{85}$Kr

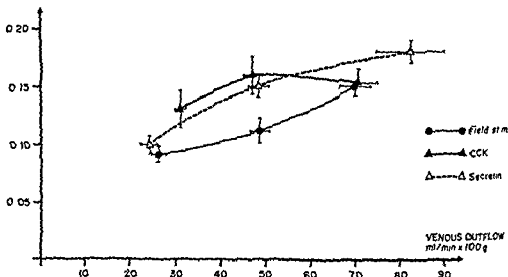



Fig 2 The relationship between total intestinal blood flow and fraction of intramural blood flow fully equilibrated with the luminal contents The blood flow rate was varied by transmural electrical field stimulation or by local infusions of cholecystokinin (CCK) and secretin Lines drawn by inspection. Bars indicate ± S E

*C Transmural electrical field stimulation* A soft plastic tube (outer diameter 2 mm) with a flattened silver wire wrapped along its length was cautiously placed inside the lumen, forming the cathode A flexible silver wire closely enwrapping the jejunal preparation served as anodal electrode. The jejunal segment was still in situ placed in a lucite chamber containing saline at 38 C (for details see Biber Fara and Lundgren 1973 a) Electrical stimulation was delivered with constant current at a strength of 60—100 mA a frequency of 60—80 Hz and a pulse duration of 8—10 ms

*D Drugs and hormones* The hormones cholecystokinin (CCK) and secretin (SEC) obtained from the Gastrointestinal Research Unit Karolinska Institutet Stockholm were given locally i a. as infusions to the intestinal preparation The cats were routinely given atropine 1 mg/kg b w ) at the start of the experiments

## Results

During control conditions the intestinal absorption rate of $^{8}$ Kr was about 5 8 ± 0 42 ml/min × 100 g (mean ± S E n = 60) at a total intestinal blood flow of around 31 0 ± 1 50 ml/min × 100 g i e the rate of absorption was similar to that earlier reported (Biber Lundgren and Svanvik 1973 c) During i a infusions (2.5—10 5 U/kg × h) to the intestine of cholecystokinin (6 expts ) or secretin (6 expts ) both intestinal blood flow and $^{8}$ Kr absorption rate increased (Fig 1) and considerably more than was the case when corresponding blood flow increases were induced by isopropylnoradrenaline infusion (Biber Lundgren and Svanvik 1973 c) This seems to be particularly true for secretin

The present technique also allowed an estimation of the fraction of intestinal blood flow that became fully equilibrated with the luminal contents which at control

blood flow amounted to 0 13±0 006 (mean±S E ) Fig 2 illustrates the results during induced intestinal vasodilatation indicating that an increasing proportion of the intestinal blood supply became equilibrated with the luminal content along with the hormonally induced vasodilatation particularly so during secretin administration

The results obtained during transmural electrical field stimulation are also given in Fig 1 and 2 showing that this procedure leads to similar results as those obtained during hormonal infusion

## Discussion

It is generally assumed that the post prandial increase in intestinal blood flow (Fronek and Stahlgren 1968 Vatner *et al* 1970) is of functional importance in meeting the increased metabolic requirements associated with increased motility and secretion but especially in increasing the absorptive capacity of the gut

To test this latter hypothesis the present study was undertaken in which the changes in rate of $^{85}$Kr absorption were investigated during the influence of such stimuli that are believed to be responsible for the functional hyperemia in the gut The results clearly support the hypothesis insofar as a close correlation was found between the rate of absorption and the extent of the intestinal vasodilator response Moreover both electrical field stimulation presumably mimicking the response to mechanical mucosal stimulation and local administration of secretin and cholecystokinin increased the rate of intestinal $^{85}$Kr absorption in excess of that seen during a corresponding isopropylnoradrenaline vasodilatation This $\beta$ receptor stimulating drug causes a vasodilatation in all intramural parallel coupled vascular circuits (Lundgren 1967 Kampp Lundgren and Sjostrand 1968 Biber Lundgren and Svanvik 1973 a) and the more pronounced increase of $^{85}$Kr absorption rate observed in connection with the functional hyperemia of the gut suggests that the mechanisms involved predominantly dilates the vessels in the absorptive mucosal parts This conclusion is further corroborated by the findings illustrated in Fig 2 showing that the fraction of total intestinal blood flow equilibrated with the luminal contents increased during the hormonally and electrically induced intestinal vasodilatations When intestinal vasodilatation is instead induced by isopropylnoradrenaline this fraction decreases with increasing total intestinal blood flow (Biber Lundgren and Svanvik 1973 c)

The present results thus suggest that a local increase and redistribution of blood flow favouring the superficial mucosal layers occurs postprandially in the small intestine Experiments to reveal the details of these hemodynamic adjustments are curently in progress using a recently developed indicator dilution technique (Biber Lundgren and Svanvik 1973 b)

In a recent publication (Biber Fara and Lundgren 1973 b) it was pointed out that intraarterially administered 5 hydroxytryptamine (5 HT) causes adjustment in the seriescoupled vascular sections similar to that observed during the vasodilator

responses induced by hormones and field stimulation Further 5 HT has been proposed to have a mediator role in the local regulation of intestinal blood flow It would therefore have been of interest to study the effect of this drug on intestinal absorption rate and attempts were also made in this direction with the present technique but it proved to be technically impossible since prolonged 5 HT infusions elicited initially intense motility increases and gradually tachyphylaxis concerning the vascular responses

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From the Department of Physiology University of Goteborg Sweden

# Pressure-Volume Characteristics of the Interstitial Fluid Space in the Skeletal Muscle of the Cat

By

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Abstract

ELIASSEN E B FOLKOW S M HILTON B ÖBERG and B RIPPE *Pressure volume characteristics of the interstitial fluid space in the skeletal muscle of the cat* Acta physiol scand 1974 *90* 583—593

Pressure volume characteristics of the interstitial fluid space in cat skeletal muscle were determined by plethysmographic recordings of the tissue volume changes following net capillary filtration or absorption. The concomitant changes in interstitial fluid pressure ($P_t$) were assessed by estimating the isovolumetric capillray pressure ($Pc_i$) In resting muscle $Pc_i$ averaged 13 7 mm Hg during perfusion with blood having an average colloid osmotic pressure of 18 3 mm Hg As there is no net fluid exchange these findings indicate a balancing filtration force of 4 6 mm Hg which is exerted chiefly by the colloid osmotic pressure of the tissue fluid ($\pi_t$) with the possible contribution of a slightly subatmospheric $P_t$

A reduction of tissue fluid volume of 30 % led to a reduction of $Pc_i$ of approximately 4 mm Hg while doubling the volume raised $Pc_i$ by 3—4 mm Hg Since these $Pc_i$ changes must be partly ascribed to alterations in $\pi_t$ the actual changes in $P_t$ were even smaller The compliance of the interstitial fluid space in skeletal muscle is therefore high (above 1 4 ml/100 g tissue × mm Hg) within the normal range of $P_t$ The large extravascular fluid depot in skeletal muscle is thus easily mobilized to replenish the circulatory system when capillary pressure falls as after a blood loss

The movement of fluid between the intra and extravascular compartment across the capillary membrane in accordance with the Starling equilibrium constitutes an important mechanism for regulation of plasma volume Of the different variables in the Starling formula the mean hydrostatic capillary pressure is the only one which can be rapidly changed by physiological adjustments to such an extent that considerable redistributions of extracellular fluid between the two compartments can quickly be achieved Such rapid and well adjusted changes in mean capillary pressure and hence in intravascular fluid content can be produced reflexly by neurogenic resetting of the pre/postcapillary resistance ratio particularly in the

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skeletal muscles (Öberg 1964), which constitute by far the most important extravascular fluid depot in the body due to their large tissue mass

The amount of fluid that can be mobilized from the muscle tissue when the capillary pressure falls is however not only determined by the magnitude of this pressure fall and by the permeability characteristics of the capillary walls for such absorption of tissue fluid will be increasingly opposed by the consequent increase of tissue colloid osmotic pressure and decrease in tissue fluid pressure both of which must occur to some extent If either or both of these forces should change appreciably as a result of small transfers of fluid the effects even of drastic reductions in capillary pressure might be rapidly curtailed As a result the large fluid reservoir present in the muscle interstitial space would be largely unavailable for effective replacement of plasma after blood loss It has in fact been suggested by Guyton (1963 1965) that small alterations of interstitial fluid volume lead to considerable changes in tissue fluid pressure within its normal range which, again according to Guyton is around $-6$ to $-8$ mm Hg This concept of a low compliance in the interstitial fluid space is based chiefly on measurements of the pressure developed in perforated capsules implanted in the subcutaneous tissue space when changes of interstitial fluid volume of a whole hind limb which is mostly muscle are being induced

On the other hand it is well known that large quantities of extravascular fluid do enter the circulation after a blood loss as reflected by the degree of posthaemorrhagic haemodilution which is often considerable Such observations as these suggest a fairly high compliance for the interstitial fluid space at least in relatively homogeneous tissues like the skeletal muscles which contain a large fraction of the total interstitial fluid volume In an attempt to resolve the apparent conflict between Guyton s results and the well known responses outlined above an analysis has been performed of the volume changes of muscle interstitial fluid that occur with known changes in mean capillary pressure or plasma colloid osmotic pressure A preliminary report of this work has been presented earlier (Eliassen *et al* 1973)

## Methods

Experiments were performed on 16 cats anesthetized with chloralose (30—50 mg/kg) The calf muscles of one leg were prepared as described previously (Djojosugito *et al* 1970) The calf was isolated from the rest of the animal except for the cognate artery and vein Three side branches of the femoral artery were cannulated one was used for measurements of the arterial inflow pressure ($P_A$) to the preparation the second for intra arterial administration of vasodilator agents and the third was connected to a reservoir containing an oxygenated dextran Tyrode solution of closely similar osmolarity to that of the original blood plasma The preparation could thus be perfused either by blood through the intact femoral artery or by dextran Tyrode solution from the reservoir The femoral vein was cannulated and the volume outflow was recorded by an optical drop chamber-ordinate recorder unit A venous side branch was cannulated for measurements of the venous outflow pressure ($P_V$) The paw circulation was excluded by mass ligatures at the level of the ankle joint. The calf was placed in a plethysmograph filled with water at 37 C and connected to a volume recording device

Before and during this preparation care was taken to avoid excess fluid accumulation in the preparation. The muscles and surrounding tissues of the limb studied were very carefully handled the main bulk of them not being touched at all The animals were at no time

primed with extra fluid The weight of the calf being investigated was compared in every experiment with that of the intact one of the other hind limb which was removed rapidly at different stages of the experiment.

The recordings of pressures flow and tissue volume changes were made on a Grass Polygraph recorder The sensitivity of the volume recorder was set so that a volume change of 0 1 ml gave a deflection of approximately 10 mm on the recorder $P_A$ could be set at any desired level either by adjustment of a screw clamp around the femoral artery (during blood perfusion) or by adjusting the height of the fluid reservoir above the preparation (during dextran perfusion) $P_V$ could similarly be controlled by adjustment of the height of the free end of the venous outflow cannula above the preparation The pressure transducers were repeatedly calibrated during the course of the experiment. Zero pressure was set at the free water level in the plethysmograph

The calf vascular bed was maintained in a state of maximal vasodilatation throughout the experiments by means of i a. infusions of large amounts of vasodilator drugs (isoprenaline 50 μg/min or/and papaverine 1 mg/min) When such vasodilatation was elicited the arterial inflow and venous outflow pressures were rapidly adjusted to levels necessary to maintain isovolumetric conditions Care was continuously taken to avoid gross changes in interstitial fluid volume unless they were deliberately induced Venous outflow was continuously recorded to check that no accidental changes of vascular tone occurred during the experiment

The capillary filtration coefficient (CFC) was repeatedly estimated during the experiment by raising both arterial inflow and venous outflow pressures by the same amount usually 5—10 mm Hg on the assumption that the capillary pressure was thereby also raised to essentially the same amount irrespective of the prevailing pre to postcapillary resistance ratio CFC was calculated from the rate of fluid filtration per unit time and tissue weight, caused by the increase in capillary pressure (see Cobbold *et al* 1963)

The isovolumetric capillary pressure i e the mean hydrostatic capillary pressure prevailing when no net fluid transfer occurred across the capillary walls was repeatedly determined by a modification of the technique described by Pappenheimer and Soto-Rivera (1948) Their method consisted of a combination of lowered arterial pressure and raised venous pressure In the present experiments $P_A$ was first raised for a short period from the level necessary for the isovolumetric state so as to cause a rise in capillary pressure and a net fluid filtration across the capillary walls After an isovolumetric state had again been established by lowering $P_A$ to the initial level $P_V$ was elevated to a level at which the same rate of fluid filtration was attained With the vascular bed maximally dilated and unable to exhibit any myogenic response the ratio between the rises of $P_A$ and $P_V$ required to produce identical rates of filtration will be very close to the ratio between the pressure drops in the pre and postcapillary vascular sections Further the potential error inherent in such methods because of the distensibility of the pre and postcapillary resistance sections will also have been minimized With the present technique both were distended together whereas in the routine employed by Pappenheimer and Soto-Rivera (1948) a passive elastic recoil of the precapillary resistance vessels appeared to have occurred coincident with a distension of the postcapillary vessels which will have resulted in an increase of the pre/postcapillary resistance ratio by virtue of the measurement procedures themselves The present modification should therefore provide a closer estimate of the prevailing pre/postcapillary resistance ratio From this ratio and with the actual figures for $P_A$ and $P_V$ obtained in the isovolumetric condition isovolumetric capillary pressure ($P_{CI}$) was calculated

The perfusion fluids used besides the cat s own blood were either a 6 % or a 4 % dextran Tyrode solution The plasma albumin and globulin contents were determined according to the method of Lowry and the plasma colloid osmotic pressure was calculated in approximate terms from the albumin and globulin concentrations measured using the equation of Scatchard (see Pappenheimer and Soto-Rivera 1948) The osmolarity of plasma and the dextran Tyrode solutions was determined with an osmometer (Advanced Instruments Inc )

## Results

*Isovolumetric capillary pressure*

Estimations of the isovolumetric capillary pressure ($P_{C_I}$) in calf muscle preparations perfused with blood was carried out in 11 experiments with complete relaxation of vascular smooth muscle all due care having been taken during the preparation and early phase of the experiment to avoid undue disturbances of the transcapillary

fluid balance (see Methods). The values for $P_{C_i}$ obtained in these muscle preparations varied between 12 and 16 mm Hg, the average being 13.7 mm Hg ± 0.43 (S.E. of mean). This is the value for the resting skeletal muscle of cat under conditions in which there was perhaps some slight dehydration as judged from the somewhat high values for plasma osmolality (usually 310—320 mOsml/l). The calculated average plasma colloid osmotic pressure ($\pi_{pl}$) in the same 11 cats based on determinations of the concentrations of albumin and globulin was 18.3 ± 0.72 mm Hg. The mean difference between $\pi_{pl}$ and $P_{C_i}$ was thus 4.6 mm Hg which must be opposed by a net filtration force of the same value in the isovolumetric state.

This isovolumetric state was usually obtained when $P_A$ and $P_V$ were set at around 30—32 mm Hg and 4—5 mm Hg respectively. This means that the corresponding pre/postcapillary resistance ratio during complete relaxation of the vessels was around 2:1 with variations between 1.8—2.3/1. For an isovolumetric $P_C$ of 13.7 mm Hg and with normal resting vascular tone and normal values for $P_A$ and $P_V$ (around 100 mm Hg and close to 0 mm Hg respectively) the pre/postcapillary resistance ratio would amount to 6—7/1.

When $P_{C_i}$ during blood perfusion was compared with $P_{C_i}$ measured during perfusion with 6 % and 4 % dextran Tyrode solutions approximately iso-oncotic with the blood, the value with 6 % dextran was found to average 15.8 ± 1.1 mm Hg, thus exceeding by a few mm Hg that obtained during blood perfusion, while with 4 % dextran it averaged 11.3 ± 0.43 mm Hg. These figures for $P_C$ indicate that the effective colloid osmotic pressure exerted by 6 % dextran across the muscle capillary walls is about 2 mm Hg higher than that of plasma with a $\pi_{pl}$ of 18.3 mm Hg as in the present experiments, or around 20—21 mm Hg. For 4 % dextran the effective colloid osmotic pressure would be about 15—16 mm Hg. These figures for [illegible] colloid osmotic pressures of the dextran solutions are considerably lower than those measured by means of *in vitro* osmometers where the 6 % dextran solution used (Macrodex, mean mol. weight 70 000, range > 90 % within 25 000—125 000) exerts a colloid osmotic pressure around 55—60 mm Hg*. One possible explanation of this discrepancy is that the smallest dextran molecules which contribute significantly to the osmotic pressure measured *in vitro* can pass rapidly across the capillary walls and therefore lead to a considerably lower intravascular colloid osmotic pressure when the more [illegible] but biologically more relevant capillary wall is used as the membrane.

In any single experi[illegible] whether measured during blood or dextran Tyrode [illegible] [illegible] 0.05 ml min$^{-1}$ 100 g during maximal dilata[illegible] [illegible] to water was not appreciably raised [illegible] during the period of pe[illegible]

* Ref. [illegible]
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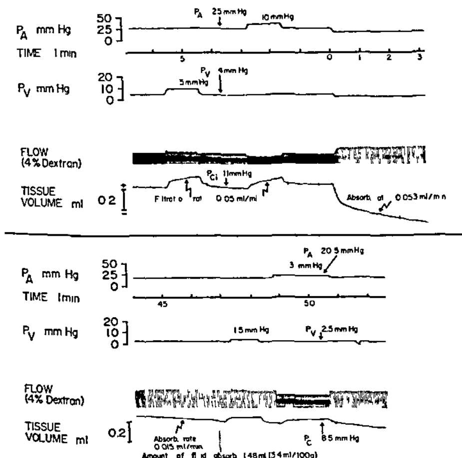



Fig 1 Records of arterial inflow ($P_A$) and venous outflow ($P_V$) pressures tissue volume changes and perfusate flow (4 % dextran Tyrode) in a calf muscle preparation In the upper panel $P_{C_i}$ is determined in the control situation by elevating first $P_V$ and then $P_A$ to such an extent as to produce identical rates of fluid filtration At time 0 a net fluid absorption is induced by lowering $P_A$ and $P_V$ —In the lower panel $P_{C_i}$ is similarly determined and found to be reduced by 2 5 mm Hg when a total amount of 3 4 ml fluid/100 g tissue had been absorbed

*Compliance characteristics of the interstitial fluid space*

Measurements of $P_{C_i}$ were performed as early as possible in each experiment after which a net filtration (or absorption) of tissue fluid was induced either by elevating (or reducing) the capillary pressure by adjusting the arterial and venous pressures or by changing the colloid osmotic pressure of the circulating fluid by switching from one perfusate to another Fig 1 shows records from one experiment where 4 % dextran Tyrode solution was used as perfusate The upper panel demonstrates the procedures for measurement of the isovolumetric capillary pressure (see Methods) which in this case was found to be 11 mm Hg at the very start of the experiment At time 0 a net fluid absorption was induced by lowering the arterial

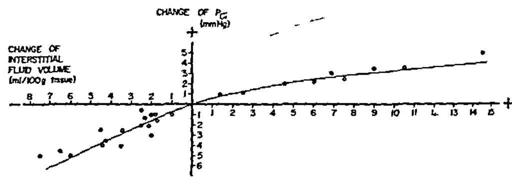



Fig 2 The relation between changes in interstitial fluid volume and $P_{Ci}$ in the calf muscle preparation Collected data from 31 determinations in 11 cats The heavy line drawn by inspection The dotted line represents the compliance curve as suggested by Guyton (1965)

and venous pressures The rate of fluid absorption was rapid at the beginning but it declined with time so that after 40—45 min (lower panel) it was only about one quarter of that found initially In the lower panel of Fig 1 the isovolumetric capillary pressure was again measured at a time when a total of 3 4 ml of fluid had been absorbed from 100 g of tissue and was found to be 8 5 mm Hg Since with a constant colloid concentration in the perfusate a transcapillary fluid equilibrium was now obtained at a mean hydrostatic capillary pressure 2 5 mm Hg lower than before there must have occurred according to the Starling principle a reduction of $P_t$ together with an increase of $\pi_{ti}$ as a result of this absorption of tissue fluid The extent to which any alteration of either of these two factors was responsible for the reduced $P_{C_i}$ was not investigated in the present experiments The change of $P_t$ however can at most equal the change in $P_{C_i}$ and this only if there were no alteration in tissue colloid pressure which is highly unlikely Therefore the fall of tissue fluid pressure following a reduction of tissue fluid volume by 3 4 ml/100 g tissue as in the experiment shown in Fig 1 must actually be less than 2 5 mm H

The relationship between the change of interstitial fluid volume measured as changes in tissue volume and the concomitant maximum possible alterations in $P_{Ci}$ from all experiments of the type described in Fig 1 is illustrated in the diagram in Fig 2 The zero point on the volume axis represents the tissue fluid volume at the beginning of the experiment No measurements of the volume of this fluid compartment were made in the present experiments it was assumed to comprise 15 ml/100 g tissue (*e g* Elkinton and Danowski 1955) The diagram shows that on this assumption the tissue fluid volume in the skeletal muscles which was reduced by 5 ml could be decreased by approximately 30 % with a concomitant reduction in $P_{ti}$ of less than 4 mm Hg as judged from the recorded change in $P_{C_i}$ and considering the increase in $\pi_t$ that must have occurred

It is also seen that when a net filtration into the tissue was induced the inter

stitial fluid volume could be doubled with only a moderate increase of the tissue fluid pressure The compliance of the interstitial fluid compartment in the skeletal muscle as indicated by the heavy line in Fig 2 thus seems to be quite high approximately 1 4 ml/100 g tissue×mm Hg within the normal range of tissue pressure In fact the true compliance is in all likelihood still higher than this figure since the interstitial colloid osmotic pressure must rise during fluid absorption and fall during filtration so that the changes occurring in tissue fluid pressure itself must be smaller than the shifts in $P_{C_i}$ actually measured The true pressure volume curve must consequently have an even flatter slope than the line in Fig 2 For comparison the compliance curve for the subcutaneous interstitial space according to Guyton s (1965) data (0 4 ml/100 g tissue×mm Hg) is also included in the diagram and indicated by the dotted line

## Discussion

The present study has shown that the isovolumetric capillary pressure in skeletal muscle of the cat is low of the order of 13 7 mm Hg This figure was obtained in experiments in which the vascular bed was perfused with the animal s own blood without the intervention of a pump and though the experiments could only be performed under conditions of maximum dilatation of the resistance vessels there was no reason to expect that capillary permeability will have deviated from normal The one qualification that should be made however is that to be precise the figure of 13 7 mm Hg refers to the hydrostatic pressure at the mid point of the system of exchange vessels and this certainly includes the immediately postcapillary section of the venules This region of equilibrium between filtration up stream and absorption down stream may be rather further down stream than has usually been considered hitherto Thus strictly speaking it is not necessarily the mid capillary pressure which is being measured indeed it is more than likely to be the mean intravascular hydrostatic pressure towards the venous end of the capillary system as morphologically defined It would perhaps be more accurate and functionally more relevant to designate the figure given above as the isovolumetric pressure of the exchange vessels This figure may then not be incompatible with that obtained most notably by Landis (1930 a and b) and more recently by Smaje Zweifach and Intaglietta (1970) for the intravascular pressure of specified capillaries measured individually under direct vision To avoid confusion in the main part of this Discussion however we will still refer to the pressure under discussion as $P_{C_i}$

The presently found value for $P_{C_i}$ may seem unexpectedly low but agrees with that reported by Diana and Kaiser (1970) for the hind limb of the dog and with the still lower $P_{C_i}$ levels observed in tissues with fenestrated capillaries where the protein content of the tissue fluid is generally somewhat higher than in skeletal muscle (intestine 9—11 mm Hg (Johnson 1965) pancreas and salivary gland 9—11 mm Hg (Eliassen Folkow and Hilton 1973))

The actual value for $P_{C_i}$ must by definition equal the sum of the other forces

which according to the Starling concept oppose capillary filtration i e $P_{C_i} = \pi_{pl} + P_{if} - \pi_{if}$ Any deviation from normal of the factors to the right in this formula will lead to a correspondingly abnormal value for $P_{C_i}$ In the present experiments the value for $\pi_{pl}$ of 18 3 ± 0 72 mm Hg is somewhat low compared e g with the figure of 19 4 mm Hg reported by Zweifach and Intaglietta (1971) in the cat This could explain in part the relatively low $P_{C_i}$ which we found If $P_{C_i}$ were adjusted so as to match the higher $\pi_{pl}$ of Zweifach and Intaglietta it would come close to 15 mm Hg As $\pi_{pl}$ in man is much higher still 23—24 mm Hg according to Zweifach and Inaglietta (1971), a correspondingly higher $P_{C_i}$ around 18—19 mm Hg might be expected in the resting skeletal muscle of this species

In the present study $\pi_{pl}$ was found to exceed $P_{C_i}$ by about 4 6 mm Hg this is considerably higher than the difference reported by Pappenheimer and Soto Rivera (1948) who found that $\pi_{pl}$ exceeded $P_{C_i}$ by only 1—2 mm Hg The values for $P_{C_i}$ (at a given $\pi_{pl}$) reported by these authors thus exceeded the values of the present experiments by 2—3 mm Hg This discrepancy may be due to differences in tissue hydration in the two sets of experiments In the present series the animals were deprived of food and water for 12 hours before the acute experiment and were if anything slightly dehydrated as may be judged from the somewhat high values for blood osmolality (310—320 mOsm/kg) As care was also taken to prevent fluid filtration into the muscle during the preparation and early phases of the experiment the fluid content of the muscle interstitial spaces was probably lower in the present experiments and $\pi_{if}$ correspondingly higher and $P_{if}$ lower than in the experiments of Pappenheimer and Soto-Rivera This could be another reason for the relatively low $P_{C_i}$ in the present experiments

The absorption force of 4 6 mm Hg which was found in the present experiments is balanced by an equal and opposite filtration force ($\pi_{if}$—$P_{if}$) under isovolumetric conditions It seems reasonable to conclude that this filtration force is exerted largely by $\pi_{if}$ The magnitude of this pressure in skeletal muscle is not known but a rough estimate may be made Under resting conditions muscle lymph usually has a protein concentration about 40—50 % that of plasma (Jacobsson and Kjellmer 1964) The protein content of the tissue fluid immediately surrounding those exchange vessels in which filtration is occurring will be decidedly lower but it can hardly be negligible since the albumin concentration in the filtrate is just a little less than 10 % of the plasma albumin concentration (Appelgren Jacobsson and Kjellmer 1966) The protein concentration in the tissue fluid surrounding the capillaries will be somewhere between these two values Gitlin and Janeway (1954) reported an interstitial fluid protein concentration in rabbit skeletal muscle of about one third that in plasma corresponding to a colloid osmotic pressure in the interstitial fluid of 4—5 mm Hg In the subcutaneous tissue of the rat Aukland and Fadnes (1973) employing the wick method found a tissue fluid colloid osmotic pressure well above one third of that in plasma but even a modest inflammatory reaction could have led to a somewhat raised protein content Even if it is assumed that only 3—4 mm Hg is exerted by the tissue fluid colloid osmotic pressure in

balance against the 4 6 mm Hg difference between $\pi_{pl}$ and $P_{C_1}$ in the isovolumetric state there remains no more than 1—2 mm Hg which can be ascribed to a negative tissue pressure The present results therefore lend no support to the concept of a markedly subatmospheric tissue fluid pressure as asserted by Guyton (1963) at least not in skeletal muscle They accord much better with the figures for interstitial fluid pressure obtained by means of the wick method (Prather Bowes Warrell and Zweifach 1971)

The most significant finding of the present study is that substantial amounts of fluid can be mobilized from, or added to the interstitial space of skeletal muscle with only small alterations of tissue fluid pressure Consequently with only small changes in $P_C$ large amounts of fluid can be exchanged between the circulating blood and this considerable fluid depot which in man is some 4—5 litres because of the large volume of skeletal muscle *In other words the interstitial fluid space in* skeletal muscle constitutes the main fluid reservoir which is readily available and can be effectively utilized for maintaining the constancy of plasma volume As however $P_C$ has to be reduced further and further in order to maintain continued absorption of muscle tissue fluid a point is reached at which no more can be absorbed because $P_t$ cannot be reduced below a finite value if circulation is to be maintained The reduction in $P_{if}$ that will also occur in such a situation and even more the increase of $\pi_t$ with increasing interstitial protein concentration will then be the factors finally bringing absorption to an end However the present results suggest that this point is not reached until a considerable proportion of available tissue fluid has been mobilized at least that part which is ordinarily held in the skeletal muscles

This finding of a high compliance of the interstitial fluid space in skeletal muscle is in contrast with the conclusion of Guyton (1965) of a surprisingly low compliance of the interstitial space at normal tissue fluid pressure According to Guyton (1965) its compliance only becomes very high when tissue fluid pressure is brought up towards 0 mm Hg or a little above which he believes to be abnormal and to result from a very small increase in tissue volume above control The discrepancy between the present results and those of Guyton cannot have been due to an abnormally high interstitial fluid volume at the onset of the present experiments so that the truly normal situation cannot be represented by the steep part of the curve (*cf* Fig 2) obtained at very low interstitial fluid volume Though the absolute volume of the muscle interstitial fluid was not measured directly in the present experiments the muscle preparations could not reasonably have been on the flat part of Guyton s curve for the interstitial fluid volume would in that case have been 6—8 ml/100 g greater and $P_{C_1}$ 4—5 mm Hg higher than normal by the time the experiment itself had started This would imply a normal $P_{C_1}$ of 8—9 mm Hg which seems far too low Furthermore when comparisons were made with the muscles of the contralateral limb even smaller weight (and hence volume increases than this were never found

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In any case the experiments of Guyton (1965) were not carried out in such a way as to justify putting all the results together as though the compliance any

particular tissue space was being measured A dog s hind limb was perfused with fluids of different colloid osmotic pressure while $P_{tf}$ was measured via a capsule implanted subcutaneously and volume changes were inferred from changes in total limb weight Below the level of frank oedema by far the greatest bulk of tissue in the limb in which the fluid volume changes will have occurred is provided by the skeletal muscles whereas the interstitial fluid pressure was measured in another compartment There is no reason to assume that $P_{tf}$ is identical at any given moment in skeletal muscle and the subcutaneous space so the two sets of figures cannot properly be plotted against one another in a compliance curve This could be an important reason for the difference between Guyton s curve and the present one It may not be the sole reason for the changes in $P_{tf}$ with variations of interstitial fluid volume which were found in the present experiments are not so different from those obtained in subcutaneous tissues when the wick method is employed (Prather *et al* 1971)

Even if some tissues may exhibit a low compliance of their interstitial fluid compartment through the normal range of $P_{tf}$ this can hardly be true of skeletal muscle in view of the well known fact that large amounts of fluid do enter the circulation after a blood loss According to the $P_{C_i}$/interstitial fluid volume relation obtained in the present study 3 ml of fluid could be absorbed from 100 g muscle (corresponding to some 900 ml from a total muscle mass of 30—35 kg in an adult man) with a fall in $P_c$ of only 2—2 5 mm Hg For fluid mobilization of this magnitude which is by no means unreasonable after a massive blood loss the figures for the tissue fluid space compliance given by Guyton (1965) would entail a fall in $P_c$ of around 10—15 mm Hg Since the level of negative tissue pressure previously suggested by Guyton already requires a very low capillary pressure around 8—10 mm Hg in the isovolumetric state at rest a capillary pressure fall of 10—15 mm Hg is impossible Even under less drastic conditions with only a moderate precapillary constriction collapse of the veins as venous transmural pressure falls will lead to a rise of postcapillary flow resistance which tends to prevent a too drastic reduction of $P_C$ It therefore seems highly unlikely that there should be a markedly negative tissue fluid pressure in skeletal muscle although it may well be more negative in the subcutaneous tissue space which differs in many respects from muscle tissue with regard to both design and function

It seems safe to conclude that the compliance of the muscle interstitial space really is high at normal tissue fluid pressures and volumes and that it is thereby well suited to function as an easily mobilizable fluid depot for the cardiovascular system

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Acta physiol scand 1974 90 594—601

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# A Microspectrophotometric Technique for Determination of Respiration in Comparison to the Cartesian Diver Method —Respiratory Activity of Rat Corpus Luteum Cells with Reference to Substrate

By

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## Abstract

Herlitz H and R Hultborn *A microspectrophotometric technique for determination of respiration in comparison to the cartesian diver method — Respiratory activity of rat corpus luteum cells with reference to substrate* Acta physiol scand 1974 *90* 594—601

A comparative study of two microrespirometric methods the classical standard-diver technique and a newly introduced spectrophotometric procedure using hemoglobin as indicator of oxygen tension as well as donor of oxygen has been performed on small samples of well-defined rat corpus luteum tissue Oxygen consumption of this tissue was determined in absence of substrate as well as in the presence of oxalacetate and succinate respectively The accuracy of the two methods was equal though small differences in absolute values were found Rapidity and simplicity of performance is considerably increased with the spectrophotometric procedure Endogenous respiration was found to be approximately 1 μl $O_2$/mg dry weight×h and in the presence of oxalacetate oxygen consumption was increased by 50 per cent In the presence of succinate respiration was increased to about 10 μl $O_2$/mg dry weight×h

The most frequently used techniques for studying oxygen consumption of small tissue samples have been micro-modifications of the classical Warburg manometric procedure Cartesian divers for determination of gas consumption or production were introduced by Linderstrøm Lang (1937) and Holter (1943) in the form of the standard diver technique having a sensitivity of $10^{-1}$—$10^{-3}$ μl $O_2$/h Another Danish scientist (Zeuthen 1953) introduced the ampulla or micro diver respirometer with *increased sensitivity* ($10^{-4}$—$10^{-6}$ μl $O_2$/h)

Spectrophotometric techniques for the determination of oxygen tension have mainly been used in the study of oxygen saturation in blood samples using hemoglobin as indicator of oxygen tension Recently kinetic macroscale studies of mitochondrial oxidative activity in dilute hemoglobin solutions have been published

(Barzu *et al* 1967, 1972) An ultra sensitive technique providing possibilities for measuring the oxygen consumption of single cells in the range of $10^{-6}$ μl $O_2$/h has been developed using hemoglobin as indicator of oxygen tension as well as donor of oxygen (Hultborn 1972)

The aim of the present study was to evaluate the accuracy and facility of a semi micromodification of the above mentioned ultra sensitive technique (Hultborn 1972) in relation to the standard-diver procedure Well-defined corpus luteum tissue from the rat was studied since its metabolism has been thoroughly investigated at our laboratory in relation to other specified cell populations in the rat ovary (Hamberger 1968 Hamberger *et al* 1971) Oxygen consumption determinations have been performed earlier on corpus luteum tissue of the rat Meyer *et al* (1945 1947) studied the succinic dehydrogenase activity of corpus luteum homogenate from pregnant rats with the Warburg technique and Channing *et al* (1966) used the same technique for studies on oxygen consumption of slices of luteinized ovaries Respiration of the same type of tissue was measured polarographically by Flint and Denton (1969) and Surwilo and Doeg (1973) In the present study pieces of one isolated corpus luteum from cycling rats were used for the studies of the oxygen uptake

## Materials and Methods

*Common procedures for both techniques*

Sprague Dawley rats 43—44 days old were used since it has been found under our experimental conditions that their first ovulation consistently occurs at the age of 41—42 days thus the tissue studied is characterized within narrow limits The rats were killed by cervical fracture The ovaries were rapidly removed and one corpus luteum was dissected free from surrounding tissue in ice-cold Tris HCl buffer 25 mM pH 7 4 NaCl 124 mM KCl 5 mM $CaCl_2$ 1 5 mM $MgSO_4$ 0 5 mM Small tissue samples corresponding to 2—30 μg dry weight were cut free from the isolated corpus luteum with the aid of a scalpel and a stainless steel needle under a Zeiss stereomicroscope

Determination of dry weight was performed after the experiment, by placing the tissue sample on a platinum foil drying at 100 C for 30 min and cooling under dry conditions in room temperature The foil was weighed with and without the sample using a balance with a sensitivity of 0 5 μg (Mettler UM6)

Three different conditions were evaluated one with substrate free buffer and the others with succinate (25 mM) and oxalacetate (5 mM) respectively as substrates The substrates were either added to the dissecting buffer preceding the spectro-photometric procedure or added directly in the diver according to a special procedure (see below) The osmolarity of the medium was kept constant by adding sodium chloride to the medium without substrate

*Standard diver technique*

The siliconized (MS 200 350 c S Midland Silicones Ltd London S W 1) cylindrical divers used in this technique suitable for measurements of gas consumption in the order of $10^{-3}$—$10^{-5}$ μl/h, are made of borosilicate glass Two dimensions of divers are used one with an internal diameter of 1 00—1 05 mm and the other 1 25—1 30 mm The gas volumes (air) of the charged divers at equilibrium pressure are approximately 7 and 13 μl respectively

Each diver is charged with 1 % (w/v) potassium hydroxide to absorb the carbon dioxide liberated by the respiring cells in the incubation medium (F[illegible] 1) Above and below the medium small drops whith substrate can be placed on the inside wall Paraffin oil (L[illegible]asol E Merck A G Darmstadt) is placed above the upper side drop to prevent exchange of material between the physiological aqueous solution in the diver and the strong salt solution (flotation medium) surrounding the diver The flotation medium in the mouth of the diver minimizes gas diffusion to or from the diver The charging of the diver is performed according to Hellerstrom (1967) where a detailed review of the above components is given by Holter & Zeuthen (1966)

The charged diver is transferred to a flotation vessel in a thermostatically controlled water

bath ($37 \pm 0.01$ C) and with a pipette the distilled water in the mouth is replaced by flotation medium The length of the mouth seal is then adjusted with a narrow tipped braking pipette (Holter 1943) until the diver equilibrium pressure is a few cm of Brodie s solution (Umbreit *et al* 1964) below atmospheric pressure The flotation vessel is then connected by a manifold to a manometer containing Brodie s solution and with compression screws the pressure is adjusted so that the diver is made to float at a certain level in the flotation medium After a temperature equilibration period of 10 minutes a positive pressure is applied to the system so that the side drop containing the substrate mixes with the incubation medium containing the tissue sample After each experiment the diver is removed from the flotation vessel and the tissue sample is recovered under a stereomicroscope by careful flushing of the upper part of the diver with distilled water from a fine pipette After determination of dry weight (see above) the oxygen consumption is calculated from the slope of the equilibrium pressure time curve according to Holter (1943)

*Spectrophotometric technique*

*Theory* The principle of the spectrophotometric procedure is to incubate the biological material to be studied in a closed chamber containing an oxyhemoglobin solution which will continuously be reduced by cellular respiration leading to oxygen removal from the incubation medium This reduction can be followed and recorded spectrophotometrically since the molecular extinction coefficients of oxygenated and reduced hemoglobin differ considerably at specific wave lengths *e g* 435 nm

Calculation of the oxygen consumption from the absorbance shift curve can be made by adding the two pools of oxygen present in the solution *i e* hemoglobin bound oxygen and physically dissolved oxygen From the slope of the steepest part of the absorbance shift curve (Fig 2) the amount of oxygen removed from the hemoglobin bound pool can directly be calculated provided the volume of the incubating chamber and the hemoglobin concentration are known Oxygen binding capacity of hemoglobin is taken as 1 39 ml $O_2$NTP/g hemoglobin. The amount of physically dissolved oxygen does not decrease linearly with time due to the presence of hemoglobin The slope of the hemoglobin dissociation curve with respect to oxygen tension should therefore be determined whenever experimental conditions are changed From the slope of this curve in the region of 50 % saturation the amount of physically dissolved oxygen per unit decrease of hemoglobin saturation can be calculated and added to th hemoglobin bound oxygen provided chamber volume and physical solubility of oxygen in the medium are known Volume displacement by the sample is corrected for when exceeding 5 % of the chamber volume For further theoretical considerations see Hultborn (1972)

*Equipment* Hemoglobin solutions are prepared from human whole blood The cells are rinsed with saline and centrifuged 3 times whereupon they are lysed by addition of distilled water Cell debris are removed as a pellet after centrifuging at 50 000 *g* for 1 h and the remaining clear hemolysate is filtered through a Sephadex G 25 column using Tris HCl buffer (see above) as eluant Taking the molecular weight of hemoglobin as 68 000 hemoglobin concentrations of up to 3 0 mM are directly obtained by fractionated collection of the effluent from the Sephadex column These solutions are kept frozen at −80 C until used To obtain the concentration of hemoglobin used in this study ($4$—$7 \times 10^{-5}$ M) the batch solutions are diluted in Tris HCl buffer and exact determinations of oxyhemoglobin concentrations are determined by the two-wave length method using accurately determined extinction coefficient (van Assendelft 1970)

A *modified Zeiss single beam microphotometer equipped* with a *monochromator* (Zeiss M4QIII and a Vitatron logarithmic recorder are used as analysing instrument Recordings are made at 435 nm using a half width of 10 nm Diameter of measured area is 45 µm. Photomultiplier RCA 1 P 28; voltage is approximately 380 V

On the object stage a temperature regulated plate is attached having connections with a thermostatically controlled water bath as well as with cold tap water Temperature is calibrated to 3 C by use of melting substances within the incubating chambers (*e g* Escotan m p 36 8 C Incubating chambers or cuvettes are produced in ordinary object slides approximately 1 mm thick by drilling a penetrating hole with a sintered carbide drill To prepare a bottom of the chamber a cover slip is fastened on to one side of the object slide by Nobecutan Bofors Nobel Pharma Molndal Sweden) sucked in between the two glasses by capillary force As a cover of the chamber an object slide of a smaller size is firmly pressed on to the other glass To be sure that the cover is tight fitting it is checked that interference fringes occur when juxtapposing the two object slides Furthermore the surfaces are siliconized (same silicone as for the divers The volumes of the chambers are determined by mechanical thickness measurements of the object slides and microscopic determination of the diameter of the hole

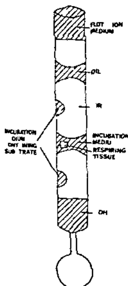



Fig 1

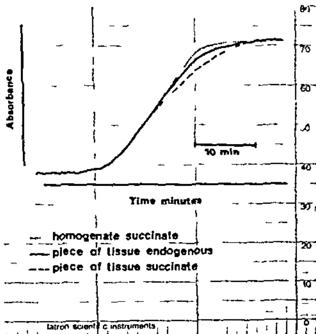



Fig 2

Fig 1 Schematic drawing of a cylindrical standard-diver The diver is filled from the bottom by means of volume-cal brated micropipettes (Modified after Hellerstrom 1967)

Fig 2 Absorbance shift curves under three different conditions Middle curve is an actual recording while the tv o others are reconstructed Observe the different approaches to the final reduced level

*Procedure* The chamber is filled by placing a large supply drop (approx. ?0 $\mu$l) of the hemoglobin solution to be used in and above the rim of the chamber The t s ue sample s placed in the supply drop and with the aid of sta nless steel needles is easily manipulated into the chamber under a stereomicroscope After thorough checking that no air bubbles have been trapped in the chamber the covering glass is placed above the chamber whereby the surplus h moglobin solution will be pressed away The preparation is transferred to the cooled object-stage and attached to it by a firm clamp (Fig 3) The preparation is centered and focused ard the measuring beam is adjusted so that it will occupy only a small fraction of the chamber area (Fig 4) Temperature regulated water is allo ed to enter the plate when the recording has commenced

*Statistical analyses*
Mean values and standard errors of the mean are g ven Comparison between different groups was performed according to Students t test. A p alu of 0 05 o less was consid red significant. Regression lines and 95 % conf dence bands for these lines have been computed accord ng to model I regress on Correlation coeff cients (r are also given (Sokal and Rohlf 1969)

## Results

*Data obtained from the standard diver procedure*
In these experiments dry weights of the tissue samples studies varied from 2 to 15 $\mu$g

The oxygen consumption of corpus luteum tissue in absence of substrate was 1 25 (55 8 nmol) ±0 15 $\mu$l O /mg dry weight×h (n = 11) In the presence of oxal

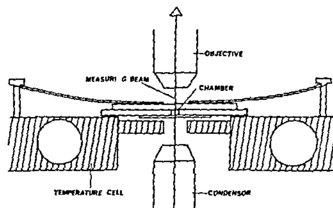



Fig 3




Fig 4

Fig 3 Section through the temperature regulated object stage with chamber in position (From Herlitz and Hultborn Acta physiol Scand 1973 87 4 A)

Fig 4 A photomicrograph of an incubation chamber with a piece of corpus luteum in hemoglobin solution Hatched area corresponds to the measuring light beam

acetate (5 mM) and succinate (25 mM) the oxygen uptake was 2 13 (95 0 nmol) ±0 37 (n = 6) and 12 51 (560 nmol) ±0 55 (n = 19) μl O /mg dry weight×h respectively This increase in oxygen consumption in the presence of the substrates is significant (oxalacetate $p < 0\ 05$ succinate $p < 0\ 001$)

No significant difference in respiration per unit weight was found between large and small samples as exemplified in Fig 5

Respiration was linear for 1—1 1/2 h without substrate and in the presence of oxalacetate In the presence of succinate respiration declined after 45 to 60 min

*Data obtained from the micro spectrophotometric procedure*

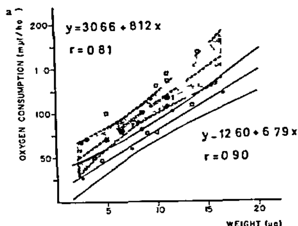
In the absence of substrate the oxygen consumption was 1 06 (47 4 nmol) ±0 04 μl O /mg dry weight×h (n = 13) using sample weights from 2 to 30 μg dry mass

In the presence of oxalacetate (5 mM) and succinate (25 mM) the oxygen uptake was 1 50 (66 7 nmol) ±0 08 (n = 12) and 8 77 (392 nmol) ±0 53 (n = 16) μl O /mg dry weight×h respectively Dry sample weights varied from 2 to 20 μg

The increase in O uptake in the presence of substrates is statistically significant in relation to endogenous respiration (oxalacetate $p < 0\ 01$ succinate $p < 0\ 001$) There was a linear relationship between oxygen uptake and dry weight under the three conditions tested as exemplified in Fig 5

In the presence of succinate the final part of the absorbance shift curves showed an earlier and more marked asymptotic approach to the final absorbance level than did the recordings under the two other conditions

No statistically significant difference between the two methods was found concerning mean values of oxygen consumption endogenously or in the presence of oxal



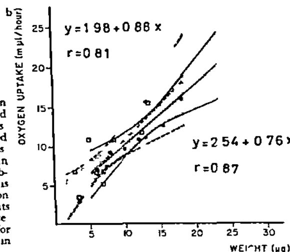




Fig 5 Regression lines for oxygen consumption versus dry weight and 95 %-confidence limits for these lines using manometric (squares shaded area) and spectrophotometric (circles dotted area) assay respectively In (a) succinate 25 mM is used as substrate whil in (b) no substrate is present in the medium Regression equations and correlation coefficients are given for the manometric procedure in the upper left parts and for the spectrophotometric technique in the lower right parts

acetate With succinate the obtained value of respiration was significantly lower ($p < 0.05$) using the spectrophotometric procedure

## Discussion

The aim of the present study was to compare the well established standard-diver technique with a newly introduced spectrophotometric procedure with respect to absolute results accuracy and simplicity and to analyse similarities and dissimilarities

There was no significant difference between the two methods concerning mean respiratory values under endogenous and oxalacetate conditions however when using succinate as sub trate a significantly lower value was obtained with the spectrophotometric procedure Furthermore there was earlier and more marked asymptotic approach to the reduced absorbance level when succinate was present

in the medium compared to the two other conditions (Fig 2) The respiratory activity of *homogenates* (unpublished data) of corpus luteum showed very abrupt approaches of the absorbance shift curve to the final reduced level (Fig 2) The abovementioned data fit well with what can be expected when considering the relatively low oxygen tension at which measurements were performed In this study the hemoglobin solutions were half saturated with oxygen at an oxygen tension of between 10 and 15 mm Hg i e the oxygen uptake is assayed at this tension or slightly above where the absorbance shift curve is steepest It is therefore reasonable to assume that there will appear gradients of oxygen tension that may lead to anoxia in the center of the sample especially in the case of high respiratory rate i e in the presence of succinate In the case of homogenates these gradients are almost nullified and therefore the asymptotic approach to the reduced level mainly represents the sigmoid relationships of the hemoglobin dissociation curve In the standard-diver procedure measurements of oxygen consumption are performed at atmospheric oxygen tension and gradients within the sample will not cause severe anoxia in the center of the tissue sample In the spectrophotometric procedure it should be considered that for small samples under slowly respiring conditions the tissue is in an environment which may show certain similarities with the *in vivo* situation concerning oxygen tension especially when considering that oxygen diffusion in liquids is enhanced in the presence of hemoglobin (Scholander 1960) Work is in progress at our laboratory to change the half saturation value of hemoglobin in this system with ligands such as 2,3 diphosphoglycerate and inositol hexaphosphate

In experiments where hemoglobin was present in the diver system no decrease in succinate respiratory activity of luteal tissue was found The lower value obtained by the spectrophotometric method using succinate as substrate can therefore not be directly due to inhibition of respiratory activity nor to an inactivation of the substrate

At the end of an experiment the incubation medium has accumulated products such as carbon dioxide leading to a slightly more acid environment compared to the original pH However it can be calculated that in the presence of the Tris-buffer and the hemoglobin this change is negligible especially since measurements of respiration are made at half-saturation of the hemoglobin i e not at the end of the running

Another parameter of interest when comparing the two methods is the degree of accuracy In Fig 5 the regression lines for sample weights versus respiration as well as the 95 % confidence limits for these lines have been presented for endogenous and succinate conditions using the two methods It is evident that the confidence limits are narrower for the spectrophotometric procedure which is best seen for endogenous respiration Under this condition it can be concluded that the accuracy of the spectrophotometric technique is greater than that of the manometric one This should be evaluated in relation to the relative ease by which the spectrophotometric chambers are charged and the automatically recording process

The accuracy of the standard-diver procedure as obtained by other investigators

(Hellerstrom 1967) is in the same range as the values achieved in this study

In conclusion it can be stated that the manometric and the spectrophotometric procedures essentially show the same results, though the accuracy seems to be some what better for the spectrophotometric method Using the latter method oxygen diffusion conditions must be considered The simplicity and rapidity (further in creased by use of automatic cuvet changing devices) of the photometric technique is higher than that of the manometric one which makes this new technique superior compared to the manometric diver procedure for oxygen consumption studies in the range of $10^{-1}$—$10^{-3}$ $\mu l$/h.

We are indebted to L Hamberger Y Kallstrom S Rosberg and A Sjogren for valuable discussions and cooperation

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Acta physiol scand 1974 90 602—608
From the Institute of Medical Chemistry University of Uppsala Sweden

# Some Chemical and Biological Properties of a Protein Fraction from Nigerseed (Guizotia Abyssinica Cass ) Soluble in Hot Aqueous Ethanol

By

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## Abstract

EKLUND A *Some chemical and biological properties of a protein fraction from nigerseed (Guizotia abyssinica Cass ) soluble in hot aqueous ethanol* Acta physiol scand 1974 90 602—608

A nigerseed protein fraction soluble in a hot water ethanol solution of sodium chloride was isolated It contained high levels of cystine and chlorogenic acid and inhibited the proteolytic digestion of casein by trypsin *in vitro* Weanling male rats failed to grow when fed on this protein fraction as the sole source of protein Histo-pathological examinations of the main animal organs did not show any abnormalities

The development of protein rich supplementary foods for weanling and pre school children requires access to protein concentrates in order to increase the protein content to about 20 % (Protein Advisory Group 1970) A protein rich raw material which attracted interest as a possible source of edible protein for use in Ethiopia was the seed of *Guizotia abyssinica* Cass in Ethiopia commonly called niger or nug (Eklund and Ågren 1970) Nug seed proteins contain most essential amino acids in satisfactory proportions with exception of tryptophan and methionine (Eklund and Ågren 1970)

A method for the preparation of a lipid protein concentrate from nug seeds has recently been described (Eklund 1971 a) By using aqueous ethanol containing sodium chloride about 70 % of the seed proteins were extracted The main protein fraction was precipitated at 60° C or more (Eklund 1971 b) The precipitate consisted of a lipid protein concentrate About 20 % of the nug seed proteins were not precipitated at 100 C and could therefore not be taken advantage of The present paper reports some chemical and biological properties of the proteins belonging to this waste fraction

## Experimental

*Preparative methods* Nug seeds from the last season s harvest were sent by air from the Ethiopian Nutrition Institute (ENI) Addis Ababa. The seeds were carefully cleaned by sieving and then pounded to a husk-containing flour in a roller mill (Artofex E. Schmid Inc., Stockholm) in which the rollers were closely set One part of the nug flour was extracted with 5 volumes of aqueous ethanol as described previously (Eklund 1971 a) The pooled extracts were cooled under tap water and centrifuged at $3000 \times g$ for 30 min at +4 C The clear oil phase floating at the top was removed The supernatant aqueous ethanol phase was concentrated to one quarter of its volume in a rotary vacuum evaporator at a temperature of about 30 C During this procedure all ethanol was evaporated In order to remove low molecular components the concentrated supernatant was dialysed against tap water for about ? days and then against refrigerated distilled water (+4 C) for 24 h Dialysis tubings 110 cm/50 ft from Union Carbide Visking Company Chicago were used after first washing with 1 M acetic acid and distilled water The dialysing process was followed by repeated conductivity measurements on the dialysate A conductivity meter type CDM 3 Radiometer A/S Copenhagen was used The content of the dialysing tube was lyophilized and finally defatted at room temperature by hexane extraction The dry material will be referred to as a high temperature soluble nug seed protein fraction abbreviated HTS-fraction *

*Analytical methods* The methods for the determination of moisture nitrogen fat content and amino acids were the same as used previously (Eklund 1971 a) Determination of chlorogenic acid was carried out on samples defatted by Soxhlet extraction with diethyl ether The phenolic acids were solubilized and separated by thin layer chromatography (silica gel coated TLC-aluminium sheets Merck product no 5553) as described by Sosulski *et al* (1972) but using absolute methanol instead of ethanol.

A standard curve was prepared by using pure chlorogenic acid (Sigma) In addition methanol extracts of the defatted samples were diluted properly with methanol and used to obtain UV absorption curves

Prior to the trypsin inhibitor activity measurements the HTS-fraction was dissolved in distilled water (1 mg/ml) under continuous stirring for 2 hrs and with pH adjusted to 8 0 by addition of 0.5 M NaOH Undissolved materials were removed by centrifugation at $1000 \times g$ for 30 min The protein content ($N \times 6.25$) of the supernatant was estimated by nitrogen analysis (Eklund 1971 b) This stock solution of the HTS fraction was diluted properly with distilled water Trypsin activity was estimated as described by Kunitz (Northrop *et al* 1948) Crystalline bovine trypsin (once crystallized) was purchased from Schuchardt Inc München. Hammarsten casein was delivered from E Merck AG Darmstadt Trypsin activity was expressed in units $[T\ U]_{Cas}$ according to Kunitz (Northrop *et al* 1948

*Animal experiments* A growth study was performed for 4 weeks under similar experimental conditions as used previously (Eklund 1971 c) 24-day old male rats of a Sprague Dawley strain were obtained from the Anticimex Farm Norrviken Sweden After the 4 week feeding period the rats were sacrificed Blood samples were taken for haematological analyses (Eklund 1971 c) Liver pancreas small and large intestine kidneys adrenals gonads spleen heart, lungs and bone marrow were examined by histo-pathological methods which have been described in a previous paper (Eklund *et al* 1971)

## Results

*Some chemical properties of the HTS fraction* The HTS-fraction accounted for 18 % of the total seed nitrogen It had a dry weight nitrogen content of 8 95 % i e a crude protein content of 56 % The fat content was 4 % of the dry weight The amino acid composition is given in Table I The most interesting observation was the high cystine content of the HTS-fraction The contents of essential amino acids were comparatively low except for cystine and methionine Chemical score calculated according to the Block and Mitchell (1946) method was 35 and threo-

4bb *reviations* HTS-fraction = High temperature soluble nug seed protein fraction TLC Thin layer chromatography T U = Trypsin units Cas = Casein.

TABLE I Amino acid composition (in mg/g N) of the HTS fraction from nug seeds Previously published data on the amino acid composition of nug seeds (Eklund and Ågren 1970) nug seed lipid protein concentrate (Eklund 1971a) and whole egg (Joint FAO/WHO Expert Group 1965) are given for comparison

| Amino acid | Whole egg | Whole nug seed flour | Nug seed lipid protein concentrate | HTS-fraction |
|---|---|---|---|---|
| Isoleucine | 415 | 307 | 341 | 201 |
| Leucine | 553 | 388 | 505 | 308 |
| Lysine | 403 | 294 | 279 | 199 |
| Methionine | 197 | 109 | 125 | 216 |
| Cystine | 149 | 177 | 97 | 537 |
| Phenylalanine | 365 | 327 | 385 | 130 |
| Tyrosine | 262 | 185 | 225 | 1 8 |
| Threonine | 317 | 237 | 263 | 112 |
| Tryptophan | 100 | 54 | 85 | 65 |
| Valine | 454 | 362 | 397 | 273 |
| Arginine | | 621 | 627 | 734 |
| Histidine | | 162 | 192 | 97 |
| Alanine | | 281 | 290 | 132 |
| Aspartic acid | | 619 | 673 | 477 |
| Glutamic acid | | 1405 | 1357 | 1911 |
| Glycine | | 375 | 357 | 295 |
| Proline | | 262 | 270 | 272 |
| Serine | | 347 | 390 | 390 |

nine was the first limiting amino acid in comparison with the pattern of essential amino acids in whole egg (Joint FAO/WHO Expert Group 1965)

As appears from Fig 1 methanol extracts of the HTS fraction as well as of defatted nug seeds show UV absorption curves which are very similar to those obtained for pure chlorogenic acid The presence of chlorogenic acid in nug seeds and the HTS-fraction is further confirmed by the TLC separation of the extracts on silica gel plates (Fig 2) One of the three bands detected under UV light had the same $R_f$-value (0 38) as chlorogenic acid The two other bands with $R_f$-values of 0 16 and 0 66 respectively might possibly correspond to *o* quinones or poorly characterized brown polymers which are formed by oxidation of chlorogenic acid (Sondheimer 1964 The contents of chlorogenic acid in defatted nug seed and in the HTS-fraction were 0 5 % and 2 0 % respectively

*Trypsin inhibitor activity of the HTS fraction* The effect of the HTS fraction on the proteolytic degradation of casein by trypsin is shown in Fig 3 There was an approximately linear decrease in trypsin activity as the concentration of the HTS-fraction increased from 40 to 170 μg per ml of incubation mixture However the specific trypsin inhibiting activity was not higher than $0\,1 \times 10^{3}$ (T U ) inhibited per μg HTS fraction The crystalline soybean inhibitor prepared by Kunitz showed 50 to 60 times higher specific activity (Northrop *et al* 1948)

*Growth studies* In order to estimate the biological quality of the HTS fraction 3 male rats were fed on a diet containing this fraction at a 10 % protein level as

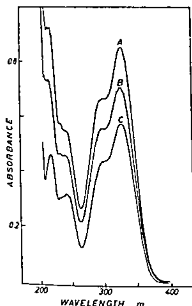



Fig 1 UV absorption curve of methanol extract of defatted nug seed (A) methanol extract of HTS fraction (B) and methanol solution of pure chlorogenic acid (C)

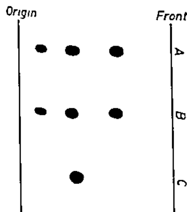



Fig 2 TLC-chromatogram of methanol extracts of defatted nug seed (A) nug HTS fraction (B) and pure chlorogenic acid (C) Silica gel coated TLC plates Eluted with n Butanol/Acetic acid/Water (4 1 5) for 4 5 h at room temperature (Sosulski *et al* 1972)

the sole source of protein Fig 4 shows the 4 week growth curve obtained with these rats and the corresponding curve obtained with a group of 10 rats fed on a reference diet based on methionine enriched casein (Eklund 1971 c) The rats on the HTS-containing diet lost 4 g during the period in contrast to the casein rats which grew normally Although the chemical score of the HTS fraction is low (35) at least some gain in body weight would be expected Consequently the growth curve obtained with this protein fraction might indicate the presence of some toxic factor(s) in the material

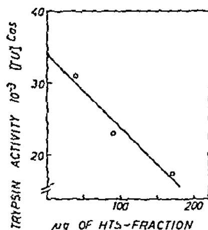



Fig 3

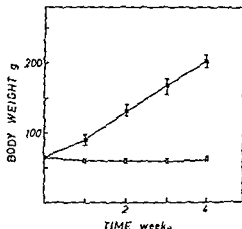



Fig 4

Fig 3 Effect of nug HTS fraction on the proteolytic digestion of casein by trypsin Trypsin activity with different amounts of the HTS fraction present per ml of 0 5 % casein and 7 5 μg trypsin

Fig 4 Growth curves obtained *with weanling* male rats fed on diets containing nug HTS-fraction (O 3 rats) and methionine enriched casein (■ 10 rats) as the sole source of protein at a 10 % protein level Mean and range values

*Organ weights haematology and histo pathological examinations* Table II gives the relative weights (grams per 100 g body weight) of liver spleen kidneys adrenals gonads and heart in rats fed on the HTS-containing diet for 4 weeks Except for slightly lower weights of the liver the values were similar to those previously found in rats of the same age fed on a methionine enriched casein diet (Eklund *et al* 1971) The histo-pathological examinations or organs showed *no abnormalities* Normal values for haemoglobin haematocrit leucocytes and thrombocytes were found in the analysis of blood sample from rats on the HTS diet (Table III) (cf Hardy 1967 Eklund *et al* 1971)

TABLE II Range of the weights in grams per 100 g body weight of organs from 3 male rats fed for 4 weeks on a diet containing nug HTS-fraction as the sole source of protein at a 10 protein level

| Liver | Spleen | Kidneys | Adrenals | Gonads | Heart |
|---|---|---|---|---|---|
| 3 04 – 3 37 | 0 23 – 0 43 | 1 09 – 1 10 | 0 076 0 073 | 0 65 – 1 77 | 0 47 – 0 57 |

TABLE III Some haematological values obtained from 3 male rats fed for 4 weeks on a diet containing the nug HTS fraction as the sole source of protein at a 10 protein level

| Haemoglobin g/100 ml | Haematocrit % | No of white blood cells per mm | No of thrombocytes per $mm^3$ |
|---|---|---|---|
| 11 9 – 13 4 | 36 39 | 4400 7400 | 344 000 – 373 000 |

## Discussion

It is essential that new sources of edible protein for human consumption should be free from toxic factors In this investigation it was demonstrated that weanling rats failed to grow when fed on a protein fraction from nug seeds This fraction also inhibited the proteolytic activity of trypsin Many experiments have shown that plant products may contain protease inhibitors which are able to depress the growth of rats and other animals (Liener 1958 Kakade *et al* 1969) The mechanism for this growth impairing effect is unknown

It is conceivable that the low nutritive quality of the HTS fraction could be explained by the presence in nug seeds of a protease inhibitor It has not been established however whether the trypsin inhibition observed with the HTS fraction was caused by a protein It has been reported that chlorogenic acid has an inhibitory effect on trypsin activity (Milic *et al* 1968) On the other hand no evidence has been presented so far that this phenolic acid induces growth depression

The high cystine content of the HTS fraction might have reduced the growth of the rats to some extent The HTS-diet used in the present experiments contained approximately 0 86 % cystine while the methionine enriched casein diet contained about 0 04 % cystine (*cf* Agren *et al* 1973) However normal growth rates of rats have been reported after the addition of 1 2 % *L* cystine to a diet containing 10 % casein (Harper *et al* 1970) In the same paper has also been reported that similar levels of cystine which reduces the growth rates produce tubular injury in the kidneys and interlobular necrosis in the liver In the present experiments no histopathological signs of tissue damage were found which might indicate that toxic levels of cystine were not reached

Nevertheless this work has shown that the protein fraction removed with the upernatant after heat coagulation of a protein concentrate from nug seeds by the method described earlier (Eklund 1971 a) is of inferior nutritional value and unfit for human or animal consumption It may be noted that protein fractions of an equally poor biological quality have been obtained from the upernatants remaining after heat coagulation of protein from extracts of fish (Hallgren 1966) and sun flower eed (Ågren 1973)

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Acta physiol scand 1974 90 609—617
From the Institute of Physiology University of Oslo Norway

# Rates of Transvascular Fluid Filtration in Lungs from Normal and Thrombocytopenic Rabbits

By

I AURSNES A HAUGE and B A WAALER

Received 18 September 1973

## Abstract

AURSNES I A HAUGE and B A WAALER *Rates of transvascular fluid filtration in lungs from normal and thrombocytopenic rabbits* Acta physiol scand 1974 90 609—617

The rate at which fluid is filtered out of the vessels on standardized elevations of left atrial pressure ($P_{LA}$) has been tested in isolated lungs from normal and thrombocytopenic rabbits Higher filtration rates were found in the lungs from normal than in the lungs from thrombocytopenic animals both during plasma and during blood perfusion The filtration rates from the two groups of lungs were significantly different both at $P_{LA}$ elevations of 10 mm Hg ($p = 0.008$ two-sided test) and at $P_{LA}$ elevations of 15 mm Hg The reason for this difference is not clear One possibility is that lungs in thrombocytopenic animals may be protected against changes occurring during the surgical procedures necessary for removal of the organ

Blood platelets do apparently influence the integrity of the wall of small blood vessels It has been known for long that permeation of erythrocytes from the intra vascular to the interstitial extravascular compartment increases considerably during thrombocytopenia (Woods *et al* 1953 Johnson *et al* 1966) Investigations by Aursnes (1974) have shown that an increased vascular permeability to albumin develops together with the increased erythrodiapedesis in thrombocytopenic rabbits Still we have very incomplete information about the vessel wall defect developing during thrombocytopenia Thus the permeability of the capillary wall for plasma constituents has not been systematically evaluated under such conditions

The present investigation is an attempt to evaluate one aspect of capillary permeability in thrombocytopenic animals namely the socalled hydraulic conductivity (Pappenheimer 1953) of the pulmonary exchange vessels The hydraulic conductivity can be defined as the rate at which filtrate moves out of the vessels upon a certain increase in transmural hydrostatic pressure This filtration rate was observed by repeated filtration tests in isolated lungs perfused with either plasma or blood containing very low number of blood platelets Organs from animals with a normal number of circulating blood platelets were compared with organs from animals where marked thrombocytopenia had prevailed for one day or more

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far end of the transducer axis was connected to a counter ballance arrangement (Fig 1) The axis of the transducer had a very low displacement on variation in load Therefore only very small changes in tension of the perfusion and ventilation tubings could occur when changes in preparation weight developed

*Perfusates* Either plasma or blood with a very low number of blood platelets were u ed *The platelet poor plasma* was obtained from blood of normal donor rabbits These rabbits were anesthetized with about 30 mg/kg of pentobarbitone (Nembutal® Abbott) i v They were then given 400 I U /kg b v t of heparin i v v hereafter blood was v ithdrawn by heart puncture To each 100 ml of blood withdrawn was added 1000 I U of heparin (pure powdered heparin Novo or Sigma) The last preparation has been shown to affect platelets to a moderate degree only (Eika 1972) This heparin preparation was therefore used in experim nts which included addition of a blood platelet suspension to the perfusate The blood v as centrifuged at 2000 x g fr 20 min at +4 C and the plasma removed Plasma v as accepted for perfusion only if the number of blood platelets was below 5000/ul If necessary a second centrifugation procedure was carreid out in order to remove additional blood platelets

*Platelet poor blood* was collected from thrombocytopenic (irradiated) donor rabbits The e donors were anesthetized heparin was given i v and blood was v itl dra n by heart puncture as described above Also to this blood heparin was added about 1000 I U /100 ml In order to get a sufficient volume of blood perfusate a small amount of platelet poor plasma was some times added ot the platelet poor blood The number of blood platelets in the blood portions u ed for perfusion was 10 000/μl or less

*Suspens on of washed platelets* in saline was prepared as described previously (Aursnes 1973) The platelets were ho ever v ashed only once

*Start of perfusion* For the first 2 min the preparations v ere perfused with a Krebs Ringer solution at 28 C in order to v ash out blood in the pulmonary vasculary bed The perfusion was then switched to either platelet poor plasma or platelet poor blood and the perfu ate temperature slowly increased to 38 C Usually some moderate admixture of the Krebs Ringer solution to the final plasma or blood perfusate took place

*Pulmonary arterial pressure* was measured from a s de tube of the pulmonary arterial cannula connected to a Sanborn P23Db transducer This transducer v as connected to one of the channels of a Sanborn model 320 dual channel DC amplifier recording unit via a Sanborn model 350—1100 C carrier preamplifier

*Weight changes* of the preparation v ere followed continuously by monitoring the signal from the force transducer via a Sanborn model 350—1100 C carrier preamplifier to the other channel of the Sanborn model 320 recording unit Calibration of the system was done at the end of each experiment by placing different weight loads on the preparation The weight increments recorded were equal to 95—100 % of the load added The total v eight of the lung with the attached heart was about 30 g and v e ght changes down to 40 mg could be detected

*Ventilation* was carried out v ith 5 % $CO_2$ in air Perfusion and inflation of the lungs v ere started simultaneously Positive pressure ventilation was initiated using a Starling Ideal pump (C F Palmer (London) Ltd ) at 22 strokes/min The inspiratory peak pressure and the minimum expiratory pressure v ere set at 10 and 1 5—2 cm of water respectively

*Filtration tests* were carried out by elevating the $P_{LA}$ by 10 mm Hg for 6 min This was achieved by forcing the blood from the outlet tubing to pass through an elevated channel in a ladder arrangement (Fig 1)

The result of such an increase in outflow pressure is an increment in preparation we ght Initially there is a marked sudden weight increase which then gradually becomes less marked After about 4 min the we ght increase continues at a steady moderate rate The first rapid increase in weight is due mainly to ascular distention itl in re nents in intra ascular blood volume (Lunde and Waaler 1969) The steady gain in weight ol s rved after 4 min is considered to be due to outwa d filtration of fluid from the small es el (Lunde and Waaler 1969) The *filtration rat is taken as the weight increase per min du ng the period from 4 to 6 min* after p essure ele ation The tests were completed afte 6 min pressure elevat on by resetting $P_{LA}$ to its initial lo value The interval between the tart of 2 su cessive tests wa chosen to be 25 min The weight of the preparation had then ret rned to the pre test level

In some experiments filtrati n tests were carr ed out at $P_{LA}$ el at on of 15 mm Hg Als n the e cases the filtration rate was recorded du ing the last 2 min of the period

The pulmonary lymph ves el we e cut in this preparation and lymph could thus escape from the lungs The extent of su h lymph e cape must ha e be n very small However s nce fluid dropped from the preparations less often than once every second min Thus escape of lymph probably did not influence the v eight increase during filtration tests

*Statistical eval ations* For the aluation of diffe nces with n each of the separate series of experiments the Wilcoxon t o sample test was u ed Th re ults from two different series of experiments were also combined in an e al at n of one particular difference se n in both se ies For this evaluation a Wilcoxon v n Eltern test an Eltern 1960) v as used

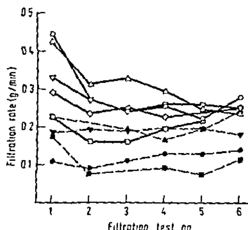



Fig 2 Filtration rates in successive standardized tests with $P_{LA}$ elevation (10 mm Hg) in 5 lung preparations from normal rabbits and in 4 lung preparations from thrombocytopenic rabbits $P_{LA}$ elevations carried out for 6 min at 25 min intervals and filtration rates measured during last 2 min of test periods as explained in text The recorded filtration rate values have been corrected for variations in perfusate protein concentration (see text) —Open symbols with thick lines results from lungs of normal rabbits Closed symbols with broken lines results from lungs of thrombocytopenic rabbits Perfusate platelet poor plasma

## Results

*Filtration tests in preparations perfused with plasma* In a first series of experiments filtration tests were carried out during plasma perfusions 5 lung preparations from normal rabbits were compared with 4 lung preparations from thrombocytopenic rabbits The first filtration test was always carried out 30 min after start of perfusion and the subsequent tests were performed at intervals of 25 min The filtration rate (g weight increment/min during the last 2 min of each 6 min test period), was usually highest in the first test carried out The subsequent 4—5 test results were fairly uniform in each single preparation and within the range of 0 08—0 3 g/min (Fig 2)

We attempted to get plasma perfusates with about the same total protein concentration in all the experiments However the plasma protein concentration in the blood of the donor animals varied to some extent Also there occurred moderately variable admixture of Krebs Ringer solution to the plasma perfusate Therefore the protein concentration of the perfusates varied between 30 and 45 mg/ml In most experiments the concentration was about 35—38 mg/ml By carrying out the standardized filtration tests ( $\Delta P_{LA}$ = 10 mmHg) at different plasma protein concentrations in 3 experiments it was found that the filtration rate increased by 0 0035 g/min for each 1 mg/ml reduction below 45 mg/ml in perfusate protein This factor was then used in the main experiments for correction of the filtration rates directly recorded according to the perfusate protein concentration prevailing at the time of each test The corrected values obtained for the successive filtration rates in the 9 plasma perfusate experiments are given in Fig 2 It will be seen that the lungs of thrombocytopenic animal tended to give lower filtration rates than did the lungs of normal animals In Table I are given the mean value of the 3 lowest filtration rates observed in each preparation These mean values were significantly different for the 2 groups of lungs (p = 0 032 Wilcoxon two-sample test (one sided))

*Filtration tests in preparations perfused with blood* In a next series of experi

TABLE I Mean filtration rates at 10 mm Hg elevations of left atrial pressure ($P_{LA}$) in 5 isolated lung preparations from normal rabbits and in 4 isolated lung preparations from thrombocytopenic rabbits The isolated lungs were perfused under constant volume inflow conditions at 38 C and with *plasma* containing less than 2000 blood platelets/μl Filtration rates were measured and corrected for plasma protein concentration different from 40 mg/ml as explained in text In each experiment 5–8 filtration tests were carried out at 25 min intervals and the mean value for the three lowest filtration rates observed in each preparation is given below When analysed with the Wilcoxon two-sample test (one sided) the difference between the given mean filtration rates in the lungs from thrombocytopenic animals and those in the lungs from normal animals was significant (p = 0.032)

| Experiment no | Lung donor | Mean value of 3 lowest filtration rates observed (g/min) | |
|---|---|---|---|
| 1 | Normal | 0.240 | |
| 2 | Thrombocytopenic | | 0.103 |
| 3 | Normal | 0.248 | |
| 4 | Thrombocytopenic | | 0.185 |
| 5 | | | 0.077 |
| 6 | Normal | 0.172 | |
| 7 | Thrombocytopenic | | 0.187 |
| 8 | Normal | 0.253 | |
| 9 | | 0.243 | |

TABLE II Filtration rates at 10 mm Hg and at 15 mm Hg elevations of left atrial pressure ($P_{LA}$) in 4 isolated lung preparations from normal rabbits and in 3 isolated lung preparations from thrombocytopenic rabbits The isolated lungs were perfused under constant volume inflow conditions at 38 C and with *blood* containing less than 10 000 blood platelets per μl In each experiment 3 or 4 filtration tests with 10 mm Hg elevation and 1 with 15 mm Hg elevation of $P_{LA}$ were carried out The individual filtration rate values were measured and corrected for plasma protein concentrations different from 45 mg/ml as described in text The mean values for the two lowest filtration rates observed at 10 mm Hg $P_{LA}$ elevation and the one value observed at 15 mm Hg $P_{LA}$ elevation are given below The differences in filtration rate values for the two groups of lungs were evaluated with the Wilcoxon two-sample test (one sided) and the p-values according to this test are given

| Experiment no | Lung donor | A Mean value for 2 lowest filtration rates at +10 mmHg in $P_{LA}$ (g/min) | | B Filtration rate at +15 mmHg in $P_{LA}$ (g/min) | | Difference B − A | |
|---|---|---|---|---|---|---|---|
| 1 | Normal | 0.185 | | 0.390 | | 0.205 | |
| 2 | | 0.163 | | 0.345 | | 0.183 | |
| 3 | Thrombocytopenic | | 0.123 | | 0.245 | | 0.123 |
| 4 | Normal | 0.140 | | 0.325 | | 0.185 | |
| 5 | Thrombocytopenic | | 0.110 | | 0.300 | | 0.190 |
| 6 | | | 0.140 | | 0.305 | | 0.165 |
| 7 | Normal | 0.210 | | 0.340 | | 0.130 | |
| Difference between two groups of lungs | | p = 0.057 | | p = 0.029 | | p = 0.31 | |

ments blood was used as perfusate Four pairs of lungs from normal and 3 pairs from thrombocytopenic animals were tested In each experiment 3 or 4 filtration tests were carried out at a $P_{LA}$ elevation of 10 mm Hg One subsequent filtration test was per

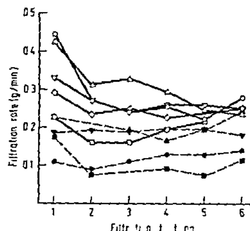



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*Filtration tests in preparations perfused with blood* In a next series of experi

ful addition of the suspensions Completely unaltered filtration rates in the subsequent 2—3 tests were then observed in these preparations The level of blood platelets had risen from 500 /μl to 38 000/μl and from 6 000/μl to 61 000/μl respectively In two pairs of lungs from thrombocytopenic animals similar careful addition of the platelet suspensions did for some reason or another lead to aggregation of platelets in the perfusate In both preparations was this followed by an increase in the perfusion pressure which with our constant flow conditions reflects an increase in pulmonary vascular resistance This increase in resistance was so marked and so long lasting in one of the preparations that reliable filtration tests could not be carried out In the other preparation in which the pressure returned to normal there was a persistent 50 % increase in the filtration rate after addition of the platelets The level of circulaitng blood platelets was at that time increased from < 1 000/μl to about 40 000/μl On further addition of platelets in this preparation spontaneous edema developed

In one of the two former preparations where filtration rates were unchanged upon blood platelet addition i v injection of 1 ml of a collagen suspension was finally carried out in order to induce platelet changes with aggregation This led to a definite increase in filtration rate (from 0 24 g/min to 0 33 g/min)

*The effect of lung interstitial water content on the filtration rate* In 1 expt with a pair of lungs from a normal rabbit we attempted to evaluate the effect of varying contents of interstitial fluid on the filtration rate at a standardized $P_{LA}$ elevation Here the intervals between the filtration tests were made to alternate between about 10 min and 30 min or more 30 min after a test the preparation would have regained its weight whereas 10 min after a pressure elevation the preparation would still weigh between 0 6 and 1 g more than before the test Most probably this additional weight reflects some remaining interstitial water accumulation The results of 6 such alternating tests are given in Table III It appears that with an increased interstitial water content the filtration rate becomes moderately reduced

## Discussion

Very little is known about the general permeability characteristics of the capillaries in thrombocytopenic individuals The purpose of the present study was to evaluate if the socalled hydraulic conductivity of small vessels is increased in thrombocytopenic animals If such a defect should be present one would suspect it to occur together with the increased erythrodiapedesis Aursnes (1973) has shown that an increased number of erythrocytes usually appeared in peripheral lymph of irradiated rabbits when the number of circulating blood platelets had been very low (about 10—20 000/μl) for abou 1 day At the same time the disappearance rate of [125]I labelled albumin from plasma increased in these animals and their weight was temporarily elevated (Aursnes 1974) These effects could be observed from day no 9 after irradiation We have used rabbits irradiated in the same way and removed their lungs on the 9th 10th or 11th day after irradiation provided the number of circulating blood platelets had been reduced to 10—20 000/μl or less for at least 24 h

The lung was chosen as a test organ because it is easy to isolate and perfuse under well controlled conditions From earlier studies we were familiar with filtration rate estimations in this organ This lung preparation can be perfused with a normal vascular resistance without spontaneous gain in weight and with maintained normal ultrastructure for several hours (Hauge and Nicolaysen 1971) We have found that half hourly repeated filtration tests are equally well tolerated in organs from thrombocytopenic and normal animals, the preparations regaining their pre test weight after each test

If functional defects develop in the capillary walls during thrombocytopenia such defects may be rapidly repaired if thrombocytes are supplied to the vascular bed (Hjort et al 1959) We therefore tested the organs from thrombocytopenic and those from normal animals during perfusion with blood or plasma with very few platelets present To our surprise we found under such circumstances that the filtration rate at elevated capillary pressure was greater in lungs from normal animals than in lungs from thrombocytopenic animals For $P_{LV}$ elevations of 10 mm Hg in the plasma perfused preparations and for $P_{LV}$ elevations of 15 mm Hg in the blood perfused preparations this difference showed a calculated level of significance with the Wilcoxon two sample test of $0.02 < p < 0.05$ (one sided tests) When the filtration rates at 10 mm Hg elevations of $P_{LV}$ in plasma and in blood perfused preparations were evaluated together in a Wilcoxon van Elteren test the difference between the filtration rates in lungs from normal and thrombocytopenic animals was highly significant ($p = 0.004$ for a one sided test $p = 0.008$ for a two sided test) It appears therefore that this difference is not accidental Contrary to our expectations we thus found a reduced hydraulic conductivity of the lung vessels in thrombocytopenic animals

Our first conclusion is that in animals with a grave thrombocytopenia lasting for 24 h or more there is definitely no increase in the measured hydraulic conductivity of the pulmonary exchange vessels However we cannot exclude the possibility that microvessels in other organs could have an increased hydraulic conductivity in such animals

What then can be the explanation for the curious finding of a reduced filtration rate in the lungs of the thrombocytopenic animals? This we do not know It appears unlikely that lack of thrombocytes should in any way tighten the lung capillaries Furthermore all rabbits were of the same initial weight with equally large lungs It therefore appears unlikely that the lungs of the thrombocytopenic animals should on an average contain a smaller capillary surface area than those of the normal animals With the dose used it also appears unlikely that irradiation should have reduced the pulmonary capillary surface area in the exposed animals (Lundborg and Schieldt 1971)

Some slight undetected degree of pre-edema may be present in the lungs of thrombocytopenic animals since such animals may show a temporary moderate increase in body weight (Aursnes 1974) With more interstitial fluid present in the lungs of the thrombocytopenic animals filtration rates on a standardized $P_{LV}$

elevation would be somewhat reduced (Table III) and our findings could be explained on this basis

It may however also be suspected that it is the filtration rate in the lungs of the normal animals which is somewhat high The permeability of the lung vessels might be altered (increased) during the induction of anesthesia or because of the surgical procedure with removal of the lungs In such a process blood platelets may be involved Adrenaline released during these conditions could well induce intravascular aggregation of blood platelets (Stehbens 1969) The platelets might be trapped in the lungs and subsequently affect the pulmonary vasculature as shown by Bo and Hognestad (1971) to occur during hemorrhagic hypotension Trapped platelets may well release substances affecting capillary permeability Our experiments on platelet addition illustrates that filtration rate actually does increase when platelet aggregation occurs with some probable release of biologically active material Thrombocytopenic animals would be protected against events of this type during the surgical procedure We regard an explanation of this type to be a likely one

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Acta physiol scand 1974 90 618—639

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# The Effect of Dopa on the Spinal Cord 8 Presynaptic and "Remote" Inhibition of Transmission from Ia Afferents to Alpha Motoneurones

By

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## Abstract

Bergmans J R. Burke L Fedina and A. Lundberg *The effect of DOPA on the spinal cord 8 Presynaptic and remote inhibition of transmission from Ia afferents to alpha motoneurones* Acta physiol scand 1974 90 618—639

In unanesthetized spinal cats injected with l DOPA, intra axonal recording was used to investigate the primary afferent depolarization (PAD) evoked in different species of afferents by volleys in the flexor reflex afferents (FRA) It was confirmed that a late PAD is evoked in Ia afferents and usually not in Ib or cutaneous afferents Some late PAD was observed in a few group II muscle afferents Intracellular recording from motoneurones revealed a considerable depression of the Ia EPSP during the late PAD evoked from the FRA after DOPA but it was difficult to attribute this entirely to the PAD in Ia afferents since there was also a late postsynaptic conductance increase in the motoneurones A further analysis was made on acute spinal cats not injected with DOPA in which a late PAD sometimes is evoked by FRA volleys without the late postsynaptic soma effects in motoneurones The marked depression of the Ia EPSP evoked from the FRA under these conditions has a longer duration than the PAD in Ia afferent terminals It is postulated that two mechanisms contribute to the Ia EPSP depression presynaptic inhibition and "remote inhibition the latter action persisting after the repolarization of Ia afferents. The results are discussed in relation to reflex regulation of stepping

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An intravenous injection of l DOPA in the acute spinal cat brings about a profound functional change in the spinal cord which includes a pattern of spinal reflexes in alpha and gamma motoneurones as well as in primary afferent terminals which is radically different from that observed in the usual low spinal preparation (Lundberg 1966 Grillner 1969 a The results of pharmacological studies support the hypothesis that DOPA acts by giving transmitter liberation from a descending noradrenergic reticulospinal pathway (Andén *et al* 1966 b Jurna and Lundberg 1968 Andén *et al* 1970 Fedina *et al* 1971)

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The present investigation is concerned with the effect evoked in primary afferent terminals from the flexor reflex afferents (FRA) after DOPA. Previous experiment with recording of dorsal root potentials (DRPs) and excitability measurements from primary afferent terminals revealed that activation of the noradrenergic pathway inhibits transmission in short latency pathways from the FRA to several types of primary afferent terminals and releases transmission in long latency pathways which produce primary afferent depolarization (PAD) in Ia afferent terminals (Andén *et al.* 1966 c). These findings have now been confirmed with intracellular recording from Ia afferents in the spinal cord. It will also be shown that during the late PAD in group Ia terminals there is a marked depression of the Ia EPSP in motoneurones but that the time course of this depression is more longlasting than is the PAD evoked in Ia afferent terminals. It is postulated that the depression is partly presynaptic and partly due to another mechanism with a more prolonged time course which is not associated with any measurable conductance change in the soma. The findings were reported in a preliminary communication (Bergmans *et al.* 1968).

## Methods

The general experimental procedure was described by Andén *et al.* (1966 a) in the first paper in this series. Recording of DRPs, excitability measurements and intracellular recording from axons and motoneurones were made as described in other papers from this laboratory. Usually the DRPs were recorded with an AC amplifier with a time constant of 1 s but DC recording was used in some critical cases (*cf.* Fig. 9 and 11). Potassium citrate micropipette electrodes were used throughout for intracellular recording.

*Abbreviations*: anterior biceps-semimembranosus ABSm, posterior biceps-semitendinosus PBSt, gastrocnemius-soleus G-S, plantaris Pl, deep peroneal (tibialis anterior and extensor digitorum longus) DP, flexor digitorum and hallucis longus FDL, hamstring (ABSm and PBSt) H, suralis Sur, contralateral co, ipsilateral i, dorsal root potential DRP, primary afferent depolarization PAD.

## Results

*1) Intra-axonal recording from primary afferent fibres*

In 6 expts. we obtained satisfactory intra-axonal recordings from 34 primary afferents of which 17 were classified as group Ia, 7 as group Ib and 2 as group II muscle afferents. The remaining 7 fibres were identified as cutaneous afferents. Differentiation between Ia and Ib afferents depended partly on threshold difference to electrical stimulation but mainly on the pattern of group I effects. It is known that Ia afferent terminals are depolarized by a train of volleys in Ia and Ib afferents from flexors but not from group I afferents of ankle extensors. On the other hand Ib afferents receive their PAD from Ib afferents of flexors and extensors (Eccles *et al.* 1962). This pattern of effects from group I afferents in Ia and Ib afferent terminals is unchanged after injection of DOPA (Andén *et al.* 1966 c). Recording was made exclusively after i.v. injection of DOPA during the period when the distinctive late DRP was evoked from the FRA. The monoamine oxidase (MAO) inhibitor Nialamid was given in some of the experiments in order to prolong the effect of DOPA (Andén *et al.* 1966 b).

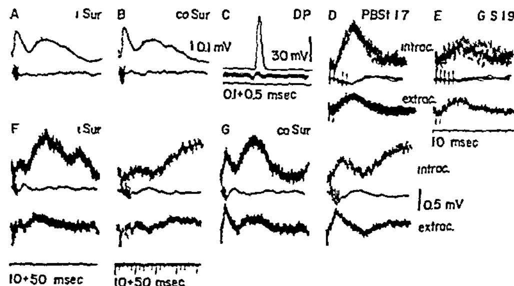



Fig 1 Late PAD evoked from the FRA in a Ia afferent fibre after DOPA 160 mg/kg i. Upper traces A and B DRPs recorded from the most caudal dorsal rootlet in L6 D—G intracellular from a DP Ia afferent (spike in C) Lower traces in A—C and middle traces in D—G are from the dorsal root entry zone Lower traces in D—G are microelectrode recordings after withdrawal of the electrode to a just extracellular position The PAD is the difference between intra and extracellular traces Left and right records in F and G were taken simultaneously at the different speeds time calibration below F A and B were taken at the same speed as left record in F Stimulus strength in D and E are in multiples of threshold for the nerve The ipsilateral (i) and contralateral (co) Sur nerve were stimulated at a strength of 20 times threshold Superimposed traces in C—E single traces in A B F G

*Ia afferents* From previous experiments utilizing excitability testing it was expected (Andén *et al* 1966 c) that a train of volleys in the FRA should evoke long latency PAD in group Ia terminals and this was indeed found in all Ia fibres tested both of extensor (G S Pl FDL ABSm) and flexor (PBSt DP) muscles

The DP afferent in Fig 1 was activated peripherally at a strength of 1 1 times threshold for the most excitable fibres in the nerve and an early PAD was evoked by a group I train from PBSt (D) whereas a train of maximal group I volleys from G S had *no effect* (E compare upper intracellular and lower extracellular traces) identifying the afferent as belonging to group Ia Records F and G show at two sweep speeds that volleys in ipsilateral or contralateral cutaneous afferent did not produce an early PAD in this fibre but instead generated a late depolarization (latency of onset about 100 ms) of about the same size as the PAD evoked by the group I train from PBSt The early DRP in A and B must reflect PAD in other fibre species (*e g* Ib and cutaneous as can be seen in Fig 2 and 3 *cf* Eccles *et al* 1962 Andén *et al* 1966 c) Similar late depolarizations were produced by stimulation of group III muscle afferents from flexors and extensors

High threshold joint afferents were not tested in the present series but the late DRP wave presumably due to a PAD in Ia afferents is evoked also by stimulation

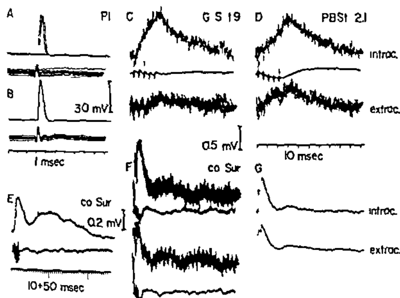



Fig. 2. As in Fig. 1 but intra-axonal recording from a Ib afferent after DOPA 100 mg/kg i.v. Spikes at threshold in A and at maximal group I strength in B. Superimposed traces in A—D, single traces in E, F. Observe slow DRP wave from co Sur in E but no corresponding late PAD in F or in G showing 10 averaged (CAT 1000) intra- and extracellular traces as indicated.

of these afferents (Anden *et al.* 1966 a). Stimulation of group II muscle afferents did not evoke any late PAD or DRP in the present series but such an effect has occasionally been encountered previously (Anden *et al.* 1966 c).

*Ib afferents.* In 6 of the 7 Ib afferents studied there was no trace of late depolarization as expected on the basis of previous excitability measurements (Anden *et al.* 1966 c). The PI fibre in Fig. 2 is depolarized by a train of group Ib volleys both from the extensor G S (C) and the flexor PBSt (D) which is the characteristic pattern for Ib afferents. A short train of volleys in a contralateral skin nerve evoked a late DRP in the L6 dorsal root filament (E) but F shows that there was no corresponding late depolarization in the fibre recorded from which is also shown in the averaged records G. Only in 1 of the 7 investigated Ib afferents did we observe a small late DRP.

*Cutaneous afferents.* The results with large diameter cutaneous afferents corresponded to those obtained from Ib afferents in that a small late depolarization was recorded only in 1 of 8 afferents. Fig. 3 C shows the presence of a late DRP with co Sur input while a comparison of the intra- and extracellular trace in D shows that there is no corresponding late depolarization in this cutaneous afferent fibre. A single volley in the SP nerve at a strength subthreshold for activation of the axon evoke a short latency PAD (B) which is invariably found in cutaneous afferents (Schmidt 1971). This early PAD is mediated by a pathway with a pure cutaneous

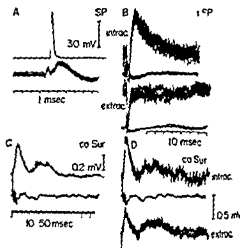



Fig 3 As in Fig 1 but intra axonal recording from a cutaneous afferent after DOPA 100 mg/kg i v Observe slow DRP wave from co Sur in C but no corresponding late PAD in D Superimposed traces in A and B single traces in C and D

input (Carpenter *et al* 1963 Schmidt 1971) and not by the pathway transmitting the short latency PAD from the FRA DOPA inhibits transmission in the latter but not in the former pathway (Anden *et al* 1966 a)

*Group II muscle afferents* It is difficult to obtain satisfactory intracellular records from group II afferents but occasional previous recordings (4 fibres) in acute spinal cats without DOPA suggested that such fibres do receive short latency PAD from the FRA (Lund *et al* 1965) as is the case with cutaneous and Ib afferents In the present series we recorded 2 group II afferents one from FDL and the other from DP Record D in Fig 4 shows that a short latency PAD was evoked in this FDL *group II afferent by a train of flexor group I volleys—this has not previously been* reported In the present context however the traces in E are of more interest since they show that a train of volleys in contralateral high threshold muscle afferents evoked a late PAD which corresponded to the late DRP produced by the same volleys (C) In the only other group II afferent in this series there was no trace of late PAD evoked from the FRA

It is of course not known if the late PAD in Fig 4 E is representative or whether it is a stray action of the type occasionally found in Ib and cutaneous afferents (*cf* above)

In view of the difficulty in obtaining satisfactory intracellular record from group II afferents it may well be more favourable to make excitability measurements on intra spinal stimulation from single afferents dissected from peripheral nerves of which the tenuissimus is the most advantageous Records F—I in Fig 4 are from a preliminary experiment of this type F and G obtained before DOPA show that a single volley in a cutaneous nerve (Sur) increased the firing index from 0/10 to 10/10 A similar effect was also evoked before DOPA by a single volley in high threshold muscle afferents (from G S stimulus strength 30× threshold not illustrated) We have thus confirmed that without DOPA a short latency PAD is

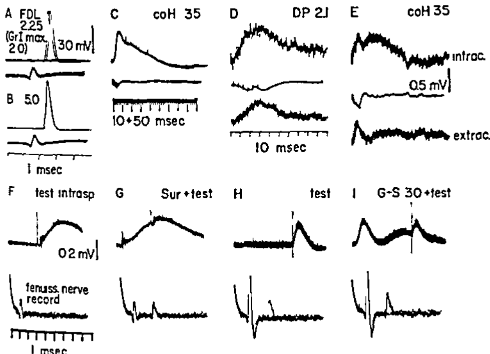



Fig 4 Records A—E as in Fig 1 but intra axonal recording from a group II muscle afferent after DOPA 100 mg/kg i v A and B spikes evoked at strengths indicated in multiple of threshold conduction velocity of fibre about 60 m/s Observe in D a short latency PAD evoked by a train of group I volleys in DP and that in E a train of volleys in contralateral high threshold muscle afferents produced a late PAD which corresponded to the late DRP hump in C Note the same time calibration for C and E Records F—I are from another experiment with intraspinal microelectrode (NaCl) stimulation 1.? mm below the surface of the cord dorsum and spike discharges recorded in the nerve to tenuissimus In all record there is an early Ia spike while the later group II spike is not evoked so regularly The fact that the Ia spike is larger in H and I than in F and G was due to electrode rearrangement Upper traces are DRPs All records consist of 10 superimposed traces F and G were obtained before H and I after DOPA 100 mg/kg i v In F the strength of intraspinal stimulation was subthreshold for the group II afferent but following a conditioning volley in the Sur 10 times threshold) the same testing stimulus invariably excited the group II fibres Similar effects (not illustrated) were evoked before DOPA by a single volley in high threshold muscle afferents and also by a short train of group I volleys After DOPA a train of volleys in higher threshold muscle afferents evoked a late DRP (I) and also an increased excitability in the group II afferent. Firing index for unconditioned and conditioned tests are given in the text Time calibration for F and G below D and for H and G below C

evoked in group II fibres from the FRA (Lund *et al* 1965) Confirming the group I effect in record D Fig 4 we also observed that a train of group I volleys in the PBSt nerve increased the excitability (the firing index increased from 1/10 to 7/10) DOPA was given after these tests and the fibre excitability tested during the summit of the late DRP then evoked from the FRA (upper trace I) The clear increase in excitability is illustrated in H and I The firing index of the unconditioned test ranged from 0—4/10 and of the conditioned test from 7—9/10 This excitability increase was smaller than that evoked via the short latency pathway

from the FRA before DOPA (F and G) and it was also smaller than the effect observed in a single Ia afferent tested in the same experiment during the summit of the late wave the firing index for the tested Ia fibre increased from 0/10 to 10/10 It is difficult to judge the significance of the moderate increase in excitability during the late DRP observed in this group II fibre and it remains to be investigated if such an effect is found in all group II afferents Our main reason for reporting this result is that it suggests an important problem which may be amenable to analysis with the method here employed, since information now is available regarding synaptic termination of group II muscle afferents in the spinal cord (Fu and Schomburg 1973)

2) *Depression of the Ia EPSP in motoneurones*

Analysis of presynaptic inhibition is often rendered difficult by the fact that the volleys producing PAD also produce postsynaptic effects Although volleys in the FRA provoke dramatic postsynaptic effects in motoneurones after DOPA there nevertheless seemed to be a possibility for an analysis of presynaptic inhibition of Ia EPSPs recorded intracellularly This possibility appeared in view of the different organization of the pathways to motoneurones and Ia afferent terminals Following DOPA late postsynaptic excitation is evoked in flexor motoneurones predominantly from the ipsilateral FRA and in extensor motoneurones from the contralateral FRA (Jankowska *et al* 1967 a) By contrast Ia afferent terminals to both flexor and extensor nuclei are depolarized from both hindlimbs (Andén *et al* 1966 c Jankowska *et al* 1966) Accordingly there seemed to be a possibility to investigate the depression of the Ia EPSP during the late PAD produced by FRA volleys not giving a late EPSP in the particular species of motoneurone recorded from Since reciprocal inhibition subserving the late reflexes is exerted mainly at the interneuronal level (Jankowska *et al* 1967 a b) it was hoped that postsynaptic inhibition in these motoneurones would not be a serious complication Unfortunately however late postsynaptic inhibition in motoneurones nevertheless proved an obstacle in the experiments performed after injection of DOPA As discussed below the most satisfactory measurements were actually made in acute spinal cats not injected with DOPA in which volleys in the FRA sometimes evoke the late PAD in Ia afferents but no apparent late postsynaptic effects in motoneurones

*Experiments after DOPA* The records in Fig 5 are from a G S motoneurone recorded after injection of DOPA (100 mg/kg i v ) A train of volleys in high threshold ABSm afferents evoked the characteristic late DRP (B C F and F lower traces and also a late EPSP in flexor motoneurones (not illustrated) The upper trace in B shows a considerable depression of the Ia EPSP approximately at the peak of the late DRP (unconditioned test Ia EPSP in A) The corresponding lower pair of records (D and F) show that although the conditioning volley did not produce any marked late PSP there was nevertheless a changed conductance at this time interval This is evidenced by the faster rise time and the lower magnitude of the membrane potential change (E) caused by a hyperpolarizing pulse (un

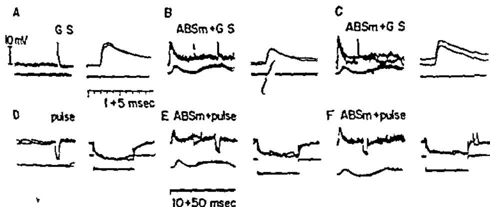



Fig 5 Depression of the Ia EPSP in a G S motoneurone evoked from the ipsilateral FRA after DOPA 100 mg/kg i v Upper traces are intracellular lower trace in A—C E and F show DRPs from the most caudal dorsal rootlet in L6 The unconditioned test Ia EPSP and test pulse (current injection through the recording electrode) are shown at two sweep speeds in A and D B C and E F show at two intervals the effects of a conditioning train in ABSm at a strength of 30 times threshold for the nerve Observe that the Ia EPSP depression is larger in B at the summit of the late DRP than in C before the onset of the late DRP while at the same intervals the depression (about 15 %) of the potential evoked by the injected current is of the same order of magnitude in E and F (or slightly larger in F) The notch at the summit of test Ia EPSP in A is probably an extracellular spike from a nearby motoneurone The records were taken 20 min after injection of DOPA at a time when volleys in the iFRA evoked large EPSPs in flexor motoneurones

conditioned pulse response in D) Since the apparent conductance change was only moderate it seemed likely that the marked depression of the Ia EPSP was not due entirely to this mechanism (postsynaptic soma conductance interaction) This interpretation is supported by the measurements in C and F in which at a briefer conditioning testing interval an equally large conductance change (F) is associated with much less depression of the Ia EPSP (C)

On some occasions the late EPSP depression was very pronounced as in Fig 6 B where the test Ia EPSP (A) is almost abolished In this case the conductance change was more marked than in Fig 5 but there seems to be a discrepancy in depression between the EPSP and the potential change evoked by the injected current In another cell illustrated in E—H there was a complete abolition (F) of a very large (3 mV) unitary (single afferent fibre) Ia EPSP evoked from the plantaris nerve Although soma conductance was not measured in the cell in E—F we find it hard to believe that even the combined effect of increased soma conductance and presynaptic inhibition could have brought about a complete abolition of the unitary EPSP Presynaptic inhibition appears to be due to a decreased probability for quantal release (Kuno 1964 b) and since the quantal content of the exceptionally large unitary EPSP in E was probably very high (*cf* Kuno 1964 a Burke and Nelson 1966 it is more likely that its total abolition may have been caused by blockage of impulse conduction in the fibre mediating the EPSP (*cf* Discussion)

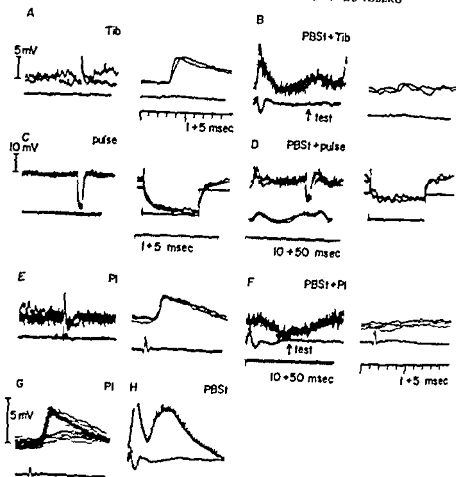



Fig 6 As in Fig 5 but with more profound depression of the Ia EPSP evoked from the FRA after DOPA 100 *mg/kg* i.v. Recording from a Tib motoneurone (A—D) and from another motoneurone receiving a large unitary Ia EPSP *from Pl* (*E—G*) Lower traces in A B F—H are from the L7 dorsal root entry zone in slow left traces in C and D and upper trace in H DRPs *from* the most caudal dorsal rootlet in L6 showing the late DRP negative wave Observe different amplitude scale for test with Ia EPSP and pulses in A B and C D respectively

We observed a parallelism between the time course of the Ia EPSP depression and the rising phase of the late DRP Another observation however made us hesitate in accepting our results as semiquantitative evidence for presynaptic inhibition We frequently found that after DOPA the recovery of the Ia EPSP depression did not have the same time course as the repolarization of the late DRP but that the EPSP depression persisted after the end of the DRP (Fig 7) Such findings necessitated a more detailed analysis in an experimental situation in which the relation between Ia EPSP depression and the late DRP evoked by FRA stimulation could be examined in the absence of complicating background postsynaptic events in the recorded motoneurone

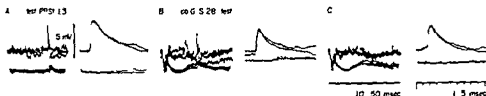



Fig 7 As in Fig 5 but with depression of the Ia EPSP in a flexor motoneurone evoked from the co FRA after DOPA 100 mg/kg i v Lower traces from the most caudal dorsal rootlet in L6 Note that the EPSP depression at the end of the late DRP is almost as large as during the summit of the late DRP

*Experiments on acute spinal cats without DOPA* Since the late postsynaptic conductance change (*cf* Fig 6 C) presumably is associated with the late reflex effects evoked from the FRA in motoneurones (Anden *et al* 1966 a) it was desirable to find a preparation in which volleys in the FRA produced late effects to primary afferent terminals without evoking late PSPs in motoneurones Jankowska *et al* (1966) reported that in some acute spinal cats without DOPA volleys in the FRA sometimes generated a late PAD in contralateral Ia afferent terminals mimicking the effect seen in DOPA treated animals This effect was however not associated with a release of transmission of late PSPs to motoneurones (unpublished observation) Accordingly we attempted to continue the analysis in preparations in which we encountered such a selective release of transmission to contralateral Ia terminals This was met with in two cats in which we recorded 8 motoneurones exhibiting no trace of late soma conductance change produced by a train of volleys in the coFRA

The records in Fig 8 (*cf* also Fig 11) are from an ABSm motoneurone recorded in one of these experiments A train of volleys in contralateral cutaneous afferents gives a late DRP (lower traces in A C F H upper trace in M) but not the late EPSP which is the characteristic effect in extensor motoneurones after DOPA (*cf* Fig 1 and 8 in Jankowska *et al* 1967 a) Observe that the early EPSP produced by the co Sur in A and B is mediated by the short latency crossed extensor reflex pathway from the FRA Transmission in this pathway is depressed after DOPA which at the same time releases transmission in the pathway mediating EPSPs to extensor motoneurones and presumably also some late postsynaptic inhibition to flexors (Jankowska *et al* 1967 a *cf* also Fu Jankowska and Lundberg to be published) Fig 8 compares at two different conditioning testing intervals (A—E F—J) the effect of a train of volleys in contralateral cutaneous afferents on the Ia EPSP (A B F G) and on the potential change evoked by a hyperpolarizing pulse (C—E H—J) The test was timed in A—E with the summit of the late DRP and in F—J to a period 100—150 ms after the apparent end of the DRP It is evident that at both time intervals there was a significant depression of the Ia EPSP (B—G) and that at both intervals this EPSP depression was not associated with any change in the apparent soma conductance (D E I J)

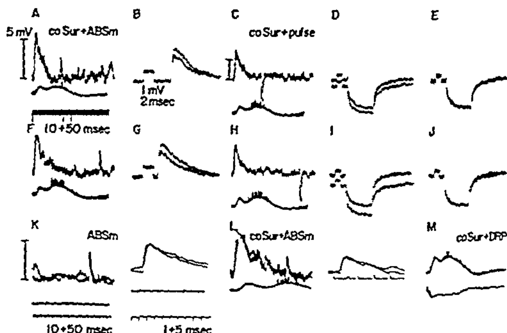



Fig 8 Experiment in which a late DRP wave was evoked from the co FRA without treatment with DOPA, with depression of the Ia EPSP from the co FRA in an ABSm motoneurone. Upper traces and averaged records are intracellular except in M Lower traces in not averaged records are DRPs. Observe that the train in the co Sur did not evoke a late EPSP in this motoneurone A C F H The early EPSP in A C F H and M is the short latency crossed effect usually found in the acute spinal cat. The two superimposed traces in B and G unconditioned and conditioned averaged Ia EPSP (10 events each alternation) condition ne test intervals are shown in the corresponding records A and F Original records at 2 speed are shown in K and L the delay is shown in low records Records C—E and H—J taken at the same conditioning test interval show no effect by the conditioning volley on the potential produced by a rectangular current pulse The traces are spaced separately in D and I and superimposed in E and J Time calibration below A refers also to C F and H and below K also to M Intracellular amplitude calibration 5 mV not lower gain in C and H

The upper curve in Fig 9 shows the time course of the EPSP depression in relation to the co Sur DRP illustrating that the duration of the former is almost twice that of the latter It has previously been shown that the time course of the late PAD in Ia afferent terminals in the motor nuclei is approximately identical with that of the late DRP Andén *et al* 1966 c) Nevertheless it seemed important to establish this relationship also under the present experimental conditions. The lower curve in Fig 9 gives the results of excitability measurements made shortly after obtaining the results shown in the upper curve Measurements from G S and PBSt are plotted together and it is immediately clear that the curve for the increased excitability runs parallel to the time course of the late DRP

We therefore conclude that the later phase (> 300 ms) of EPSP depression is not due to a depolarization in Ia afferent terminals but to another mechanism. As regards the nature of this mechanism the first possibility to consider is that the mere existence of a longlasting PAD of any kind in some way has after-effects lead

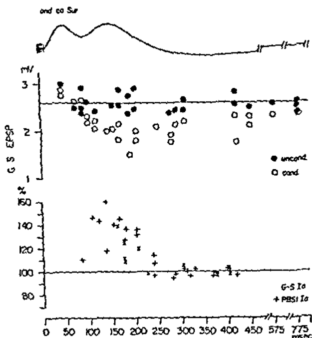



Fig 9 Same experiment as Fig 8 Upper graph gives the EPSP depression in relation to a tracing of the DRP recorded with DC amplification The corresponding lower graph shows excitability measurements from G S and PBSt Ia afferents ad modum Wall (1958) The terminals were stimulated in the respective motor nuclei with a low resistance NaCl glass microelectrode and the antidromic volleys recorded in the appropriate muscle nerve 100 % on the ordinate is the amplitude of the unconditioned antidromic test response Observe correspondance in DRP and excitability decay but longer persistance of EPSP depression

ing to decreased transmitter output In order to test this possibility we investigated the Ia EPSP depression evoked by a longlasting train of group I volleys Fig 10 illustrates the characteristic finding that after the end of stimulation there is a swift recovery of the Ia EPSP depression with a time course that roughly parallels that of the DRP Accordingly it seems likely that the late depression in Fig 8 and 9 is not caused simply by the preceding PAD no matter how prolonged but is rather the result of another action evoked from the FRA

The rising phase of the curve of Ia EPSP depression does seem to run in parallel with the PAD in Ia afferents as reflected by the DRP or terminal excitability increase but Fig 9 suggests that the maximal EPSP depression is delayed with respect to the DRP summit and this dissociation appears even more clearly in Fig 11 where the DRP was maximal after about 200 ms while the maximal EPSP depression occurred after aproximately 300 ms We envisage that the onset of the Ia EPSP depression is caused largely by the presynaptic depolarization of Ia terminals that the portion of EPSP depression not due to PAD has a slow onset and that the two effects add to give maximal Ia EPSP depression during the later part of the PAD

Since the two mechanisms giving Ia EPSP depression seem to occur together both in experiments with and without DOPA it is likely that they in some way are linked However the fact that they have different time course suggests a certain degree of independence between the two processes

Eide *et al* (1968) postulated that the depression of the Ia EPSP evoked by a

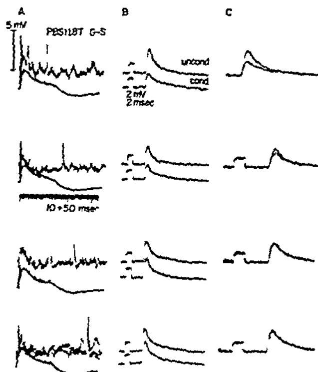



Fig 10 The recovery of Ia EPSP depression evoked by longlasting stimulation of flexor group I afferents The averaged (10 events each alternating) Ia EPSP in B and C were taken at conditioning test intervals shown in the records in A in which lower traces are DRPs The PBSt nerve was stimulated at 200/s and the duration of the stimulation (about 250 ms) appears from the DRP trace

train of volleys in group I afferents from flexors is due entirely to presynaptic depolarization The reason for this conclusion was not only that the process of EPSP depression has the same time course as the depolarization in Ia afferent terminals (Eccles *et al* 1961) but also that the depressed Ia EPSP has the same shape measurements as the unconditioned test Ia EPSP The latter finding does not seem to be compatible with the possibility of postsynaptic inhibition in distal dendrites originally discussed by Frank (1959) As regards the mechanisms of the late Ia EPSP depression from the FRA which is apparently not caused by a PAD it is of interest that there appears to be some change in the shape of the depressed EPSP In Fig 8 the conditioned EPSP (G) has a slightly shorter time to summit and a somewhat faster decay than the unconditioned EPSP (G) and there is evidence of a faster decay also in C Fig 11 Corresponding slight changes were observed in 3 of the 6 motoneurones in which the time course of the Ia EPSP could be evaluated These changes may be the outcome of an increased inhibitory conductance at sites in distal dendrites (often called remote postsynaptic inhibition) As discussed in detail by Rall (1967) detection of dendritic conductance changes by observing the distortion in voltage transients produced by current pulses from an intrasomatic electrode is difficult since the sensitivity of the method decreases very sharply with increasingly distal sites of conductance change In view of the slight effects and the small material it is clear that further investigations are required to find out whether or not dendritic inhibition is a significant part of the mechanism giving the late Ia EPSP depression from the FRA

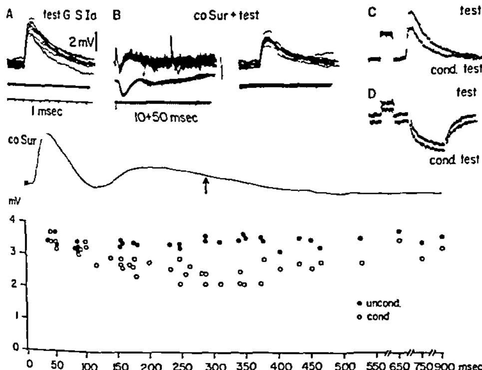



Fig 11 As in Fig 8 but with recording from a G S motoneurone Note DRP trace in B reversed in polarity with negativity downwards DRP above graph traced from DC record Arrow indicates conditioning testing interval at which the averaged records in C and D were taken The graph shows more clearly than the one in Fig 9 that the maximal Ia EPSP depression occurs after the summit of the DRP

## Discussion

We have confirmed with intra axonal recording that after an injection of DOPA volleys in the ipsi and contralateral FRA produce a longlasting depolarization in Ia afferent terminals (Anden *et al* 1966 c Jankowska *et al* 1966) There is usually no corresponding depolarization in Ib afferents or in cutaneous afferents Our finding thus support the conclusion (Anden *et al* 1966 c) that volleys in the FRA which in the acute spinal cat depolarize their own terminal after DOPA instead produce a depolarization primarily in Ia afferent terminals As an explanation of this switch in pattern Anden *et al* (1966 c) proposed that the release of transmission to Ia afferent terminals was secondary to an inhibitory action from a noradrenergic reticulospinal system on the short latency reflex pathway from the FRA The inhibition of the latter pathway was supposed to remove an interactive inhibition which normally prevents transmission from the FRA to Ia afferent terminals

Our material of group II afferents is clearly too small to allow a definite conclusion but the finding that a late PAD was evoked from the FRA after DOPA in

? of the 3 investigated fibres suggests the possibility that the late PAD may not be confined to the terminals of primary spindle afferents but perhaps is also evoked in terminals of secondary spindle afferents This possibility has important implications since these two afferent systems have entirely different segmental effects

*The mechanism of Ia EPSP depression*

It is generally assumed that PAD in a given set of synaptic terminals gives presynaptic inhibition (Eccles 1964) and we aimed at finding how the monosynaptic Ia EPSP in motoneurones was influenced during the late PAD evoked in Ia afferents from the FRA It was difficult however to obtain decisive results from intracellular recordings made after injection of DOPA The reason was that the profound depression of the Ia EPSP produced by volleys in the FRA after DOPA occurred together with a late postsynaptic conductance change (*cf* Fu Jankowska and Lundberg to be published) A quantitative analysis required a testing situation in which the conditioning volleys did not evoke postsynaptic effects in the soma of the recorded motoneurone As noted earlier we made use of the finding that in acute spinal cats without DOPA the characteristic late PAD sometimes can be evoked in Ia afferents from the co FRA without an associated late reflex effect in motoneurones Under conditions with no evident change in soma conductance (and *little or no change in background synaptic activity) we found that* the Ia EPSP depression considerably outlasted the late DRP Although it might be argued that the DRP reflects a PAD in more dorsally located Ia afferent terminals and that the depolarization of the terminals in the motor nuclei could be more longlasting our excitability measurements revealed that the time course of the PAD in the latter terminals was identical with that of the late DRP (Fig 9 *cf* also Andén *et al* 1966 c)

In previous investigations the PAD and Ia EPSP depression evoked by a short train of group I volleys had the same time course (Eccles *et al* 1961) However in view of the longlasting PAD in the present circumstance the question arose whether the depression of Ia transmitter release might outlast the presynaptic depolarization if the latter is sufficiently prolonged Our results (*cf* Fig 10) show *that this is not so in the case of presynaptic inhibition of the* Ia EPSP evoked by longlasting group I trains from flexors We therefore conclude that even when there is no conductance change observable at the soma the late depression of the Ia EPSP evoked by volleys in the FRA after DOPA is produced *both by presynaptic depolarization and by another mechanism*

Frank (1959 *originally proposed that the depression of the* Ia EPSP by group I volleys from flexors could be either presynaptic or caused by a postsynaptic inhibition exerted on dendrites at some distance from the soma The Ia EPSP is generated by terminals widely distributed over the soma and dendrites of motoneurones (Burke 1967 Eide *et al* (1966) argued that remote postsynaptic inhibition could not be responsible for the depression of the Ia EPSP by group I volleys from flexors because the depolarization occurred without any change in the time

course of the Ia EPSP An IPSP in distal dendrites would shorten the time course of the composite Ia EPSP recorded in the soma through a selective depression of the electrotonically slowed components generated at distal dendritic sites It is thus important in the present results that during the late depression the Ia EPSP may have a somewhat shorter time to peak and faster decay even when the apparent conductance measured in the soma did not increase Accordingly we cannot exclude that a conductance change in distal dendrites contributes to the persistent depression which occurs after the repolarization in Ia afferent terminals Below we will utilize Frank s term remote inhibition when referring to the depression of the Ia EPSP over and above that evoked by depolarization of Ia afferent terminals We use "remote as a descriptive term of convenience and our proposal that it may be associated with postsynaptic inhibition of distal dendrites is very tentative Furthermore remote inhibition will be discussed as a mechanism controlling transmission from Ia afferents but it is not yet known if this action is a selective one or whether it also affects other excitatory system converging on the motoneurones

Vyklicky *et al* (197?) have recently shown that stimulation of the sciatic nerve increases extracellular $K^+$ in the rat spinal cord The authors raised the question whether the increased $K^+$ may be responsible for PAD and presynaptic inhibition (*cf* Barron and Matthews 1938) In our opinion many findings make it very unlikely that the short latency PADs are evoked by such a mechanism (*cf* Eccles 1964 Lundberg 1966 Schmidt 1971) It is more difficult to exclude the possibility that increased extracellular $K^+$ may be responsible for the late long lasting PAD evoked from the FRA after DOPA The late reflex excitation of motoneurones is mediated by interneurones located just dorsomedially of the motor nucleus (Jankowska *et al* 1967 b) Very longlasting high frequency discharges in these interneurones are evoked from the FRA after DOPA Ia fibres en route to motoneurones traverse or pass close to this interneuronal nucleus (Szentagothai 1967) Potassium ions leaking from the highly active interneurones might affect the Ia afferents while Ib and cutaneous afferents terminating more dorsally would not be affected Furthermore it has recently been shown that group II afferents have one of their terminal regions very close to or even within the motor nucleus (Fu and Schomburg 1973) Accordingly a $K^+$ leakage might affect also group II afferents which would tally with the preliminary observations discussed above The hypothesis that the late PAD is produced by $K^+$ leakage from the interneurones mediating the late reflex effects to motoneurones is attractive since it would provide a simple common explanation for a variety of findings Nevertheless there are two sets of observations that oblige us to adhere to the original hypothesis that the action is exerted via a pathway with axo-axonic synapses on Ia afferent terminals (Anden *et al* 1966 c) First as mentioned under Results in some acute spinal cats the late PAD can be evoked by volleys which do not evoke late discharges (Jankowska *et al* 196[illegible]) or late EPSPs in motoneurones (Fig 8 and 11) Since in this case the establishment of the late PAD seems to be dissociated from transmission in the reflex pathway to motoneurones we infer that the PAD is not a secondary consequence of the activation of this pathway Second after DOPA a corresponding late PAD is evoked from the FRA also to Ia afferent terminals on DSCT cells (Jankowska Jukes and Lund 1965) Since these afferents pass rostrally 3—5 segments via the dorsal column to reach Clarke's column it is in this case virtually excluded that the depolarization is due to increased extracellular $K^+$ in the interneuronal nucleus referred to above

The same argument (i e dissociation from activation of the reflex pathway to motoneurones can be utilized to postulate that the very late remote depression of the Ia EPSPs is not due to $K^+$ leakage Admittedly increased $K^+$ in the extracellular space of the distal dendrites might give an EPSP depression without changed soma conductance but an increased $K^+$ would be expected to depolarize also the Ia afferent terminals on these dendrites and the excitability measurements from Ia afferent terminals failed to reveal such a late depolarization (Fig [illegible])

We do not wish to exclude that increased extracellular $K^+$ may play a role within the late reflex pathway to motoneurones activated after DOPA A depolarization due to increased extracellular $K^+$ may add to the transsynaptic mechanisms discussed above to give further depression of the Ia EPSP We have reported one case of a probable conduction block in a Ia

afferent evoked from the FRA after DOPA (*cf* Fig 6) We should not entirely disregard the possibility that a conduction block in Ia afferents en route to motoneurones might be produced as a conjoint effect of the depolarization caused by increased extracellular K and the electronic spread from the terminals

*The functional role of the Ia EPSP depression*

In order to appreciate the functional significance of the present results they must be discussed in relation to the main effect evoked from the FRA when the descending noradrenergic system is activated by an injection of DOPA the late longlasting discharge in α and motoneurones with predominant excitation of flexors from the ipsilateral FRA and of extensors from the contralateral FRA (Andén *et al* 1966 a Jankowska *et al* 1967 a b Grillner *et al* 1967 Bergmans and Grillner 1969 Grillner 1969 b) The interneurones mediating these effects to the motoneurones are organized in a half centre fashion with mutual inhibitory connexions between the interneurones exciting flexors and extensors (Jankowska *et al* 1967 a b) This interneuronal network can give alternating activation of extensors and flexors and has been assumed to function as a *spinal locomotor centre* which may be utilized also in the intact animal (Jankowska *et al* 1967 a) In this connexion it is of considerable interest that activation of the noradrenergic system which releases transmission from the FRA to this interneuronal network induces acute spinal cats to walk a treadmill (Budakova 1971 Forssberg and Grillner 1973 Grillner 1973) Furthermore results from recent investigations suggests that the noradrenergic reticulospinal system is activated by stimulation in the mesencephalic locomotor region which produces stepping in high decerebrate cats (Grillner and Shik 1973 Grillner 1973)

The parallel actions to α and γ motoneurones from the FRA after DOPA have been taken to indicate that the spinal locomotor centre operates with α γ linkage (Grillner 1969 a Lundberg 1969) as found during stepping in the mesencephalic cat (Severin *et al* 1967) Besides activation of the spinal locomotor centre volleys in the FRA evoke the following effects after DOPA

1) Depolarization of Ia afferent terminals giving presynaptic inhibition of transmission from Ia afferents (*cf* above)

2) *Remote inhibition giving longlasting inhibition of Ia transmission to* motoneurones (*cf* above)

3) Depression of transmission in the recurrent inhibitory pathway from motor axon collaterals Bergmans *et al* 1968)

4) Facilitation of transmission in the reciprocal Ia inhibitory pathway and postsynaptic inhibition in motoneurones evoked via Ia inhibitory interneurones (Fu Jankowska and Lundberg to be published)

All these subsidiary actions are concerned with transmission *from* Ia afferents and point to the importance of the regulation of the stretch reflex apparatus in stepping The actions under 3) and 4) presumably regulating reciprocal Ia inhibition are discussed by Fu Jankowska and Lundberg (to be published) and we

will now consider the functional role of 1) and 2). In a preceding paper in this series Grillner *et al.* (1967) mentioned regulation of the degree of Ia excitation and interruption of the Ia–γ loop as two possible roles for presynaptic inhibition. We will discuss mainly the former mechanism but also briefly consider the latter possibility in relation to gallop.

Grillner *et al.* (1967) found late activation of static γ motoneurones evoked from the FRA after DOPA and discussed the possibility that the depolarization of Ia afferent terminals may tend to bring about a switch from a Ia to a group II loop which might have the role of maintaining the late discharges (*e.g.* activation of the spinal locomotor centre). Subsequent investigations, however, have shown that dynamic γ motoneurones are activated as well during the late discharge (Bergmans and Grillner 1969, Grillner 1969 b). The very fact that both types of γ motoneurones are activated emphasizes the role of primary spindle afferents and accordingly suggests that presynaptic inhibition and remote inhibition of transmission from Ia afferents is related to a regulation of the actions evoked from these afferents.

Assuming that the γ-operated stretch reflex gives servo assistance in movement (Matthews 1972) it seems possible that a regulation may be required during locomotion because load compensation through the stretch reflex should be compatible with certain features of the normal step which may be influenced by the stretch reflex. It has been suggested that the requirement for a longlasting yield in extensors (2nd extension phase) should be considered in relation to reflex regulation of the step (Lundberg 1969). The basis of this hypothesis was the observation (Engberg and Lundberg 1969) that the yield contributes to keeping the body level during locomotion (in walk and trot *but not in gallop*). Stretch of the activated muscle spindle during the yield evokes a powerful Ia discharge (Severin *et al.* 1967). If sufficient Ia excitation of motoneurones was added to the direct α route depolarization extensor activation might get strong enough to elevate the body of the animal in the midst of the stance (a wasteful expenditure of energy). It is clear that for this purpose the yield should not be considered alone—a distinct separation in the 2nd and 3rd extension phase is actually only found in gallop, while during slow progression there is almost a plateau in the middle of stance with very little angular changes at the knee and ankle or in the length of the muscle which extends at these joints (Engberg and Lundberg 1969, Goslow *et al.* 1973). The maintenance of a level body during stance may require a delicate balance—varying with the speed of locomotion—between direct α route excitation and indirect γ loop Ia excitation. To some extent such a balance may be built into the activation of the spinal locomotor centre by the relative amount of α and γ activation but presynaptic and remote inhibition could also play a role as regulator of the amount of Ia excitation reaching motoneurones.

Activity in the FRA probably increases with the speed of locomotion (*cf.* Lundberg 1973). If so, inhibition of transmission from Ia afferents may also increase giving a decreased proportion of indirect γ route Ia excitation to direct α route

excitation with increasing speed of locomotion However in this connexion it is of interest that the inhibitory mechanisms controlling Ia excitation can be activated by volleys in the FRA under conditions when these volleys do not evoke late reflex actions in motoneurones suggesting some functional independence of the pathways mediating the former actions from those activating the spinal locomotor centre and from its activity Thus there may also be the possibility of an independent regulation for example from higher centres of the mechanisms modulating transmission from Ia afferents making it difficult to predict how these mechanisms will operate during locomotion in the intact animal

During high speed gallop the duration of the yield may be as brief as 31 ms (Goslow *et al* 1973) Grillner (1972) has postulated that because of the long loop time stretch reflex autoregulation does not occur during the stance phase of high speed gallop and that load compensation in this case is entirely a muscular event During the stance phase in high speed gallop there is a very large yield in knee and ankle extensors (for example the soleus is stretched 16 mm at the very high rate of about 0 5 m/s Goslow *et al* 1973) This displacement must give a powerful well synchronized discharge in Ia afferents and yet during the yield in gallop there seems to be a plateau in the EMG activity without any obvious phase of activity adding to that starting about 10 ms before the placing of the foot (Engberg and Lundberg 1969) Assuming that motoneurones are available for recruitment under these conditions it may be asked whether presynaptic and remote inhibition of transmission may be powerful enough to suppress any additional Ia activation of motoneurones? Even a reduction of the Ia EPSP to half would hardly be expected to bring about a complete suppression of the stretch reflex considering that the motoneurones are presumably effectively depolarized also via the direct α route However it remains to be investigated whether blockage of Ia fibres en route to motoneurones by increased extracellular K (cf small print section above) may also play some role Postsynaptic (soma) inhibition of motoneurones from the contracting extensors should also be considered For example extensor Ib afferent must be very effectively activated during the stance in gallop and if the interneuronal transmittability is appropriate Ib inhibition may balance any increased Ia excitatory action reaching the motoneurones

*Ib inhibition* to extensor motoneurones might also contribute to the mechanism which keeps the body level in slower types of locomotion Lundberg 1969) suggested that Ib inhibition may be one factor assuring a longlasting yield by preventing its early termination through the Ia stretch reflex mechanism If one would desire an even balance between Ia excitation and Ib inhibition in the beginning of stance but a shift in favour of Ia excitation later on during the stance in order to allow the stretch reflex to function as a load compensating reflex in progression Apart from a possible difference in primary afferent activity there is actually a mechanism which may bring about such a shift We are referring to presynaptic inhibition of transmission from Ib afferents evoked by impulses in Ib afferents (Eccles *et al* 1962) Since this process has a relatively slow onset it may provide a

negative feed back control of Ib inhibition with a built in delay and thus gradually decrease the efficacy of transmission in the Ib inhibitory pathways to extensor motoneurones during stance

In contrast to the above presumed temporal role of presynaptic inhibition of transmission from Ib afferents in one phase of the step we rather assume that the presynaptic and remote inhibition of transmission from Ia afferents evoked from the FRA because of the long duration and wide receptive field (*cf* Anden *et al* 1966 c) are equally effective in the swing phase and during stance It is well to remember that this discussion has been limited to two of the presynaptic inhibitory mechanisms which may operate during stepping and that so far no attempt has been made to give any functional role to the well known short latency presynaptic inhibition of Ia transmission evoked mainly from flexor Ia and Ib afferents (Eccles *et al* 1961)

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Acta physiol. scand. 1974. 90. 640—644

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# Effect of TRH, and Short-term Exposure to Experimental Stress or Cold on the Serum Immunoassayable TSH Concentration in the Rat

By

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## Abstract

LEPPÄLUOTO, J., T. RANTA, H. LYBECK and R. VARIS. *Effect of TRH and short-term exposure to experimental stress or cold on the serum immunoassayable TSH concentration in the rat.* Acta physiol. scand. 1974. *90.* 640—644.

The influence of TRH, transfer and sampling stress and cold on the blood TSH level was studied in female rats using a specific radioimmunoassay for rat TSH. The maximum response to TRH increased with the dose of TRH and occurred at 5 min with 10 and 50 ng. With a TRH dose of 250 ng the maximum response occurred at 10 min. Transfer stress caused a significant rise in serum TSH level at 5 min. After this the serum TSH level gradually decreased and the decrease at 30 min was significant compared with 5 min value. Sampling stress caused a slight increase in the serum TSH level at 5 min and at 30 and 60 min the serum TSH level was significantly lower than in controls. The maximum effect of cold on the blood TSH level was seen in animals kept for 30 min at 5 °C. These studies clearly demonstrate the effect of TRH on the serum TSH level in rats. The activation of the secretion of TSH in the cold is physiologically relevant, and the inhibition of TSH secretion in response to stress refutes the hypothesis that stressful stimuli activate the thyroid gland.

It is evident that the effects of various environmental stimuli on the activity of the thyroid gland can, at the present time, be estimated most precisely from the changes in the serum thyrotrophin (TSH) concentration. In several previous studies the effects of exposure to stress and cold on TSH secretion have been established by estimating TSH biologically or indirectly in experimental animals (Brown-Grant *et al.* 1954, Kraicer *et al.* 1963, Jobin and Samel 1964, Ducommun *et al.* 1966) and thus these data do not represent actual blood TSH concentrations. With the development of sensitive radioimmunoassays for measurement of this hormone a more accurate re-examination of the effects of various environmental stimuli on the pituitary thyroid axis has been possible.

In this paper we report the effects of TRH administration and short-term exposure to cold or stress on the serum TSH level in rats by means of a sensitive radioimmunoassay.

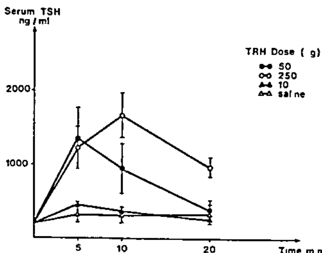



Fig 1 Effects of varying amounts of TRH (0 10 50 250 ng i v ) on serum TSH concentration in female rats

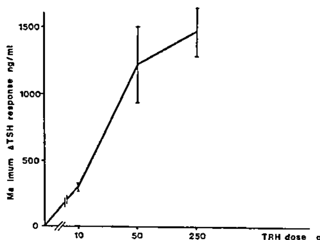



Fig 2 TRH dose response curve Each point represents the mean of the maximum increas s in TSH (maximum ΔTSH) in response to the indicated TRH dose in ten rats Vertical lines indicate ± S E TRH dose is plotted on a log scale p values of dif ferences 10 ng versus 50 ng ($p < 0.05$) 50 ng versus 250 ng (not significant) 10 ng versus 250 ng ($p < 0.01$)

## Materials and methods

*An mals diet and housing*

Sprague Dawley rats altogether 78 females weighing 180—280 g were fed *ad l b* on a pelleted diet (iodine concentration about 0 6 mg kg and tap water The animals were kept in cages (1—5 per cage) and were not handl d on the exper mental day Th room temperature was 20—22 C and no artif c al illumination v as used The cages were so arranged that a cage could be taken out of the room without disturbing th other rats

*Experimental design*

The effect of TRH administration was studied as follows 20 female rats were anesthetiz d with pentobarbital (40 mg/kg) 30 min later a 0 5 ml blood sample v as drawn by ca diac puncture and then saline or 10 50 or 250 ng TRH was injected int a femoral vein ach to 5 animals Blood samples were then taken similarly at 5 10 and 20 min

The effect of short term exposure to stress on the erum TSH l el was studied n t o experiments 1) 5 rats were decap tated and blood was coll ct d with in 1 min after the observer entered the room 5 other animals were transferred to an adjacent room for 5 15 or 30 m n and handled as above 2) blood as collected by hea t puncture from 10 rats at

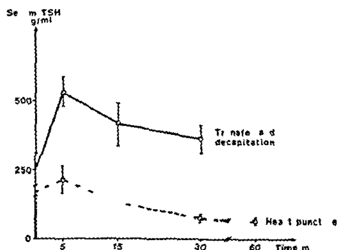



Fig 3 Effects of transfer and sampling stress on serum TSH level in female rats

1 min of the observer s entry The heart was punctured again at 5 30 or 60 min so that each animal was bled 2 or 3 times

The effect of short term exposure to cold on the serum TSH level was studied in 2 expts 1) 5 rats were transferred in their cages to each of 4 rooms with temperatures of 20 15 10 and 5 C 30 min later the animals were decapitated and blood collected 2) 20 rats were transferred to a room with a temperature of 5 C and 5 animals were taken out and handled as before at 15 30 45 and 60 min

Serum TSH was estimated individually from duplicate 200 µl samples by a specific double antibody radioimmunoassay A rat TSH kit was received as a gift from the NIAMD The results were expressed in ng/ml of a NIAMD TSH RP 1 standard

Student s t test was used for comparisons

## Results

### *The effect of TRH administration on the serum TSH level*

The serum TSH concentration rose significantly after injection of 10 ng of TRH (Fig 1) The maximum response increased with increasing dose of TRH and occurred at 5 min with 10 and 50 ng With a TRH dose of 250 ng the maximum response occurred at 10 min When a dose response curve was plotted from the above data it was found (Fig 2) that the curve fell somewhat at the dose of 250 ng

### *Short term exposure to experimental stress and serum thyrotrophin*

The effect of transfer stress is shown on Fig 3 In control animals the serum TSH level was 249 ± 58 ng/ml (mean ± S E ) and in the animals taken out of the animal room for 5 min TSH was significantly increased to 530 ± 52 ng/ml ($p < 0\ 01$) The serum TSH level was 411 and 360 ng/ml in the animals exposed to transfer stress for 15 and 30 min respectively The decrease from 530 to 360 ng/ml was also significant ($p < 0\ 05$)

In the following series of experiments 10 animals were subjected to heart puncture The serum TSH level was in the control animals (heart puncture within 1 min) 168 ± 18 ng ml and had risen to 213 ± 49 ($p > 0\ 05$) on repuncture at

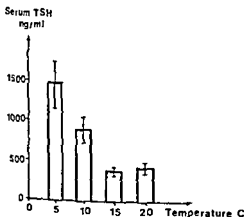



Fig 4

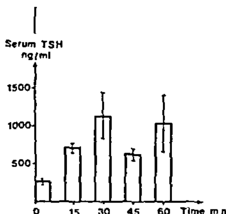



Fig 5

Fig 4 Effect of transfer to various temperatures on serum TSH level in female rats

Fig 5 Effect of exposure to cold for various time intervals on serum TSH level in female rats

5 min At 30 and 60 min the serum TSH fell significantly to 74 and 64 ng/ml ($p < 0.01$) (Fig 3)

*Short term exposure to cold and serum TSH*

Transfer of the animals to 10 and 5° C significantly increased the serum TSH level from 426 to 894 ($p < 0.05$) and to 1478 ng/ml ($p < 0.01$) as seen in Fig 4 and 5 The serum TSH level was unchanged when the animals were transferred to rooms with temperatures of 20 or 15 C In the following experiment it was seen that TSH was significantly increased within 15 min after transfer to 5° C The maximum increase was seen at 30 min ($p < 0.01$) (Fig 4)

## Discussion

In this study the TSH response to synthetic TRH appeared to be dose dependent between the doses of 10 ng and 250 ng in anesthetized animals Similar results have been reported earlier by others (Gordon *et al* 1972) In high doses (*i e* 250 ng) the maximum TSH response occurred later and seemed to be proportionally lower than expected These findings enabled us to plan experiments in which the TSH response to TRH was used as an indicator We recommend that in such cases 25—50 ng of TRH should be given iv and blood samples drawn at 0 5 and 10 min

Two quantitatively different nociceptive stimuli transfer stress and sampling stress elicited here a biphasic response in the secretion of TSH a rapid increase at 5 min and a decrease at 30—60 min In previous studies of this kind exposure to transfer or injection stress has been found to result in a monotonous decrease in plasma TSH level (Kraicer *et al* 1963 Ducommun *et al* 1966 The initial increase

of the hormone level found here was not great and the bioassay used by those authors may have failed to detect this phenomenon Although the behaviour of the TSH response to transfer stress resembled that seen after sampling stress it is pointed out that transfer stress did not significantly decrease the serum TSH level as did the sampling stress probably owing to the different intensities of transfer and sampling stress The decrease in the serum TSH concentration in response to stress appears to be the rule in the rat but is significant according to the present results after 30 min

In these test animals the serum TSH level increased with the intensity of cold (maximal effect at 5° C) and was significantly elevated after 15 min with a maximum at 30 min these results are in accordance with a similar study (Jobin and Samel 1964) We have obtained similar results also in male rats (unpublished observation) Close comparison of our results with those of this earlier study is not possible because exact information was not available

The rapid increase in serum TSH level suggests that neural mechanisms are involved in the response to short term exposure to cold in the rat We emphasize that although exposure to reduced environmental temperature is known to stimulate the pituitary thyroid axis demonstrations of increased serum TSH levels in response to this stimulus have been successful only in rats (Jobin and Samel 1964 Ducommun *et al* 1966 Hershman *et al* 1970 Lybeck *et al* 1973) and in human infants (Wilber and Baum 1970) Thus cold exposure in the rat offers an experimental model in which the interactions between hypothalamus adenohypophysis and thyroid can be studied

In response to stress or cold clear and rapid changes in the serum TSH level were observed in the rat The activation of the secretion of TSH in the cold is physiologically relevant and the inhibition of TSH secretion in response to stress shows that stressful stimuli do not activate the thyroid gland

We are grateful to the Rat Pituitary Hormone Distribution Program NIAMD NIH for providing the rat TSH RIA kit.

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Acta physiol scand 1974 90 645—647

From the Department of Clinical Physiology Karolinska Hospital and the Department of Physiology Gymnastik och idrottshögskolan Stockholm Sweden

# Glycogen Content of Individual Muscle Fibres in Man

By

BIRGITTA ESSEN and JAN HENRIKSSON

Human skeletal muscle contains two major fibre types fast twitch (FT) and slow twitch (ST) The PAS stain has been used to evaluate glycogen content in these two fibre types (For ref see Gollnick *et al* 1972 ) Histochemical techniques can however at best be semi-quantitative and it was therefore of interest to develop a method to determine the glycogen content of individual muscle fibres in a quantitative way Such a method must at the same time permit the classification of the individual fibres with respect to fibre types

## Methods and procedure

Muscle biopsies were taken from the lateral portion of the M quadriceps femoris of one subject at rest, at different times during a 2 h work bout on a bicycle ergometer and 5 h post exercise Each biopsy sample was divided into three portions and immediately immersed in liquid nitrogen The portion used for glycogen analysis of individual fibres was freeze dried (Leybold Heraeus GT 2 GFR) and placed under a dissection microscope (40×) With special instruments 80—100 individual fibres 2—3 mm long could then be dissected out The freeze-dried fibre when exposed to ambient room air (27 C 30 % relative humidity) will take up $O_2$ $N_2$ and water equivalent to about 5 of its weight After that the weight of the fibre is constant (Lowry and Passonneau 1973) This was checked by weighing the same fibre at different times during the day

Each individual fibre was then treated as follows

1 The fibre ends were stained for myosin ATPase at pH 9 4 for identification of fast twitch (FT) or slow twitch (ST) characteristics (Padykula and Herman 1955)

2 The weight of the remaining portion of the fibre was obtained with a quartz fibre fish pole balance fluorometrically calibrated with quinine hydrobromide The weights averaged 1 8 (0 4—3 0)

3 The fibre was placed in a 100 μl capillary tube and 8 μl of 1 M HCl was added After sealing the tube it was hydrolysed for 2 h at 100 C

4 Two to three μl of the hydrolysed solution was added to 150 μl of reagent solution and analysed for glucose residues according to the Lowry method (Karlsson Diamant and Saltin 1970)

The remaining two portions of the muscle sample were analysed for total glycogen content (Karlsson Diamant and Saltin 1970) and histochemically stained for myosin ATPase and glycogen (Padykula and Herman 1955 and Pearse 1961)

## Results and discussion

At rest the glycogen content of the FT fibres averaged 525 (129—938) mmol glucose units×kg⁻¹ dry weight The corresponding value for the ST fibres was 51 (142—919) Approximately 80 % of both the FT and ST fibres had glycogen

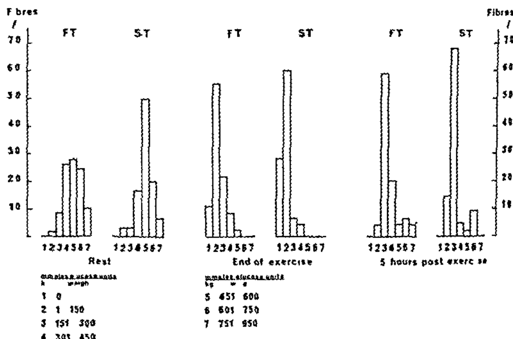



Fig 1 Distribution of the glycogen content of the individual fibres at rest end of exercise and 5 h post-exercise The number of fibres in each class is expressed in percent of the total amount of fibres of each type analysed (Because two fibres had a glycogen content above 900 mmol × kg⁻¹ class 7 is extended to 950 )

tents between 300 and 750 mmol × $kg^{-1}$ (Fig 1) After 40 min of exercise the mean glycogen content was 214 (43—521) mmol × $kg^{-1}$ for the FT and 222 (4[illegible]—4[illegible]5) mmol × $kg^{-1}$ for the ST fibres After 80 min of work the glycogen content was further reduced and averaged 156 (0—683) for FT and 135 (0—493) mmol × $kg^{-1}$ for ST fibres respectively At the end of the 2 h work bout the glycogen content of the FT fibres averaged 152 (0—549) and that of the ST fibres 71 (0—433) mmol × $kg^{-1}$ Twenty per cent of the fibres showed no glycogen content at all Five hours after exercise following a carbohydrate rich meal the values were for FT 275 (124—978) and for ST fibres 259 (60—678) mmol × $kg^{-1}$ respectively The distribution of the glycogen concentrations of the individual fibres is indicated in Fig 1

The relationship between glycogen content determined on muscle homogenates (independent variable) and mean values of individual fibres gave the following equation

$$y = 0.99\,x + 1.2 \quad r = 0.99 \quad SD = \pm 4.3 \text{ mmoles} \times kg^{-1} \text{ w.w.}$$

Fibres weighing 0.3—3.1 $\mu g$ were divided for glycogen determination on each half and the error of the method given as the *coefficient* of variation of a single value (SD of the difference between the fibre halves

$$\times \frac{1}{\sqrt{2}} \quad \frac{100}{\text{mean value of glycogen content}}\Bigg) \text{ was } \pm 6.2\,\%$$

When the fibre weights are as low as 0 5 μg with a low glycogen concentration, the error can be up to ±50 mmol×kg^-1 dry weight These latter findings then demonstrate that the present method determines glycogen in individual fibres accurately

During and after exercise the glycogen distribution pattern estimated from the PAS stain showed a roughly similar picture to that obtained from individual fibre analysis Both methods demonstrate that the two fibre types contain a similar amount of glycogen at rest However it is evident that at rest with ample glycogen stores there is a wide variation of glycogen content within both fibre types which can not be observed when using PAS stain The fact that PAS stain is too insensitive at high glycogen contents makes a direct determination preferable

This study was supported by a grant from the Swedish Medical Research Council (40X 2203)

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# Stimulation of Adenosine 3, 5-Monophosphate Formation in Mast Cells by 5-Hydroxytryptamine and Guanethidine

By

J Gripenberg M Harkönen and S E Jansson

Evidence is accumulating to support the view that changes in the intracellular concentration of adenosine 3 5 monophosphate (cAMP) are closely related to the mediation of the various effects elicited by biogenic amines in peripheral tissues (Sutherland and Robison 1966) and in the central nervous system (see e g Rall and Sattin 1970) Transmitter kinetics and the mechanism of action of various drugs on uptake and release of monoamines have been studied using *in vitro* models for the aminergic nerve terminal including blood platelets (Sneddon 1973 and refs therein) and mast cells Rat mast cells besides passively incorporating histamine (Cabut and Haegermark 1966) also take up 5 hydroxytryptamine (5 HT) by an apparently active carrier mediated mechanism located at the cell membrane (Jansson 1970) and the same mechanism seems at least partially to account for the accumulation of guanethidine in these cells (Gripenberg 1973) The objectives of the present study were twofold firstly to test whether the mast cell could serve as a suitable model in the study of the mechanisms of amine induced cAMP formation and secondly to investigate the correlation between the amine carrier and *receptor sites responsible for stimulation of cAMP formation* This was achieved by challenging the cells with guanethidine 5 HT and histamine and by observing the changes induced in the concentration of cAMP and the activity of adenyl cyclase *The results presented indicate that mast cells possess an amine* sensitive cAMP system and that in these cells there is an intimate correlation between the amine carrier sites and the receptor sites involved in the induction of cAMP formation

Isolated peritoneal mast cells from Sprague Dawley rats of both sexes were used The procedures for washing out isolating and counting the mast cells as well as the composition of the Tris-HCl buffered electrolyte solution used for incubations have been described earlier (Gripenberg 19 3 [illegible] ))[8] Pharmacia in saline served as gradient material Washed and pooled mast cells were incubated in plastic tubes in duplicate at 37 C in a water bath for 0 3 7 and 15 min respectively The incubation volume was 300 μl with 2 5×10[4] mast cells/sample After preincubation for 5 min at 37 C the agents under study were added No drugs were added to zero time samples Incubations were terminated by the addition of 3 0 ml ice-chilled medium to the tubes After centrifugation at 750×*g* for 10 min the supernatants were aspirated and the pellets frozen in liquid nitrogen and stored at −70 C prior to th

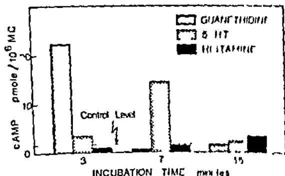



Fig 1 Effect of guanethidine 5 HT and histamine on the accumulation of cAMP in isolated mast cells *in vitro* Guanethidine at $5\times10^{-5}$ M and 5 HT and histamine at $10^{-4}$ M were added after 5 min of preincubation at 37 C Incubations were carried out for 3 7 and 15 min The dotted line represents the basal level of cAMP in mast cells Each bar represents the mean value of 2 expts in duplicate Ordinate Amounts of cAMP as pmol/$10^6$ mast cells (MC) Abscissa Incubation time in minutes

determination of the cellular contents of cAMP and enzyme activities cAMP was measured by radioimmunoassay (Radioimmunoassay kit Schwarz/Mann Co ) after precipitation of proteins and Dowex resin column chromatography of the supernatants Adenyl cyclase and cyclic nucleotide phosphodiesterase activities were measured according to the method of Harkonen *et al* (1974) after freezing and thawing and sonication of the mast cell pellets Protein measurements were performed according to the method of Lowry *et al* ([illegible])

The basal level of cAMP in isolated mast cells was $1\,33\pm0\,33$ pmol/$10^6$ mast cells corresponding to $6\,78\pm2\,25$ pmol/mg protein (mean and S E of 5 determinations in duplicate) The adenyl cyclase activity was $32\,2\pm8\,5$ pmol/mg protein/min at 30° C while the phosphodiesterase activity was some 10 times higher [illegible] pmol/mg protein/min at 30 C Guanethidine at $5\times10^{-5}$ M and 5 HT at $10^{-4}$ M were potent stimulators of cAMP formation while histamine at $10^{-4}$ M was less effective in this respect (Fig 1) Guanethidine caused an almost 20 fold transient increase in the cAMP level of mast cells in 3 min The effect of 5 HT at $10^{-4}$ M was slightly different since maximal amounts of cAMP were recorded after 7 min of incubation In one experiment however the highest amounts of cAMP were found after only 3 min with 5 HT The increase in cellular cAMP levels induced by guanethidine or 5 HT both returned to control levels when incubations were carried out for a full 15 min probably reflecting increased hydrolysis of cAMP by phosphodiesterase Yet preincubation of mast cells with guanethidine for 15 min did not abolish the responsiveness of the system to 5 HT When added 15 min after guanethidine 5 HT caused a new pronounced increase in the cAMP level indicating the possible existence of multiple activating sites for the system involved in the formation of cAMP

When added simultaneously guanethidine and 5 HT produced a less marked response and the cAMP peak found after 3 min with guanethidine alone was depressed when 5-HT was present The very slow formation of cAMP observed after incubation with histamine may indicate that there is a close correlation between amine carrier sites and the receptor site(s) responsible for the activation of cAMP formation As phosphodiesterase activity was not measured in mast cells incubated in the presence of drugs the changes observed in adenyl cyclase [illegible] are not easily interpreted The rise in cellular cAMP levels found after [illegible]

with guanethidine and 5 HT was however accompanied by a rise in adenyl cyclase activity whereas histamine was ineffective in this respect The exact site of action of guanethidine and 5 HT on the cAMP system in mast cells cannot be elucidated on the basis of the present preliminary results It is however conceivable that besides competing for the same amine carrier sites (Gripenberg 1973) which as judged from the present results are intimately associated with adenyl cyclase enzyme(s) in the mast cell membrane guanethidine and 5 HT also influence phosphodiesterase activity possibly in different ways In conclusion mast cells might serve as a valuable *in vitro* model for the monoaminergically influenceable cAMP system allowing the use of very short incubation periods and eliminating such drawbacks of slice techniques as diffusion of agents to and from the activating sites

This work was in part supported by grants from the Medical Research Council in the Academy of Finland and the Sigrid Jusélius Foundation Helsinki Finland

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# Effects of Substance P on the Response of Guinea Pig Vas Deferens to Transmural Nerve Stimulation

By

U S von Euler and P Hedqvist

The smooth muscle stimulating and vasodilating agent found in extracts of intestine and brain (known as Substance P (SP) (Euler and Gaddum 1931)) was isolated in 1970 by Chang and Leeman from extracts of bovine hypothalamus shown to have a high content of this substance (Pernow 1953) Chemically it proved to be a straight-chain undecapeptide (Chang Leeman and Niall 1971) After its synthesis (Tregear *et al* 1971) it has become available for biological testing

In the present study we wish to report some actions of synthetic SP on the response of the isolated guinea pig vas deferens to field stimulation and its interactions with some drugs known to affect the mechanical responses of this organ Enhancing effects on the nerve stimulus response of this organ have been reported for a variety of substances including crude SP (Sjostrand and Swedin 1968)

Guinea pig vas deferens was dissected out and placed in a 5 ml bath with Tyrode solution (for composition see Hedqvist and Euler 1972) aerated with 5 % $CO_2$ in $O_2$ Field stimulation was applied through platinum electrodes in the wall of the bath Postganglionic nerve stimulation was checked by recording the response after addition of hexamethonium and guanethidine

The organ was stimulated every minute with 25 biphasic pulses applied through a Grass S4 Stimulator at 5 or 10 Hz in order to elicit the twitch Pulse duration was as a rule 1 ms Isotonic contractions were recorded by means of a transducer coupled to a Honeywell 190 inkwriter using a load of 0 25—0 5 g Synthetic Substance P was kindly placed at our disposal by Professor S Leeman and used in aqueous solution of 1 or 10 µg/ml

In concentrations from 0 1 ng/ml SP regularly enhanced the initial twitch contraction of the guinea pig vas deferens elicited by transmural stimulation (Fig 1) Occasionally a clearcut enhancement could be demonstrated with as little as 250 fg/ml When comparison was made between crude and chemically pure SP one unit corresponded to approximately 5 ng of the pure compound While the effect of a given dose of SP showed little variation on repeated administration an increase of the dose augmented the response only moderately Moreover in some cases the effect of SP although generally marked remained the same over a wide range of doses suggesting an all or none character of action An elevation in the basal tone of the preparation was a regular feature even with small doses (Fig 1)

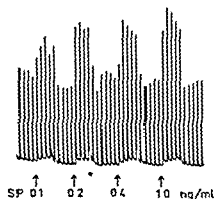



Fig. 1. Contractile responses of the guinea pig vas deferens to transmural stimulation (25 pulses 10 Hz 1 ms supramaximal voltage) resumed each min. Substance P (SP) added at arrows. Wash at dots.

The increase in the mechanical response to transmural stimulation observed with SP was relatively greater at 5 Hz than at 10 Hz when a total of 25 pulses were delivered. At shorter stimulus duration (0·2—0·3 ms) the effect of a given dose of SP was also as a rule more marked than at a longer stimulus duration (1—2 ms).

Phentolamine in doses ranging from 0·02 to 2 μg/ml bath fluid commonly increased the "twitch" response to transmural stimulation. This effect, which was usually noted only after the first addition, is presumably due, at least in part, to the abolition of an inhibitory α effect on nerve transmission, as evidenced by the increased release of noradrenaline from the transmurally stimulated guinea pig vas deferens even after pharmacological blockade of NA uptake mechanisms and of

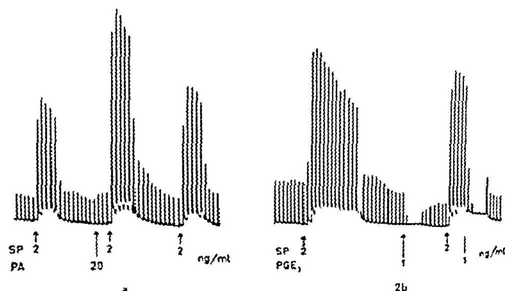



Fig. 2. Contractile response of the guinea pig vas deferens to transmural stimulation (25 pulses 1 ms supramaximal voltage 10 Hz left panel, 5 Hz right panel) resumed each min. Substance P (SP), phentolamine (PA) and prostaglandin $E_1$ ($PGE_1$) added at arrows. Wash at dots.

local prostaglandin formation (cf Hedqvist 1973) After a previous dose of phentolamine (0 02 μg/ml) the enhancing effect of SP on the twitch was further augmented (Fig 2) Correspondingly the effect of SP was reduced by the α agonist clonidine which by itself reduced the twitch response The inhibition of the twitch response caused by $PGE_1$ (Hedqvist and Euler 1972) still occurred after enhancement of the twitch by SP (Fig 2)

The twitch response of the guinea pig vas deferens is normally inhibited by PGEs (Hedqvist and Euler 1972) Similarly the twitch can be inhibited by low doses of NA although it is usually moderately enhanced by high doses (Holman and Jowett 1964) The weak stimulant effect of NA in conjunction with several other findings led Ambache and Zar (1971) to question the role of NA as muscle activating transmitter in this organ

The present observations suggest that SP like bradykinin enhances the stimulus response in the vas deferens by partial depolarization of the muscle membrane (Sjostrand 1973) This contention is further supported by the increase in tone observed in this preparation even with concentrations of SP as low as $10^{-13}$ M The remarkable potency of SP together with its occurrence in subcellular particles in adrenergic nerves (Euler 1963) raises the question of SP as a possible physiological mediator of smooth muscle activity

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# Analysis of Design and Reactivity of Series-coupled Vascular Sections in Spontaneously Hypertensive Rats (SHR)

By

B Folkow M Hallbäck Y Lundgren L Weiss I Albrecht[1] and S Julius[2]

In a series of studies (*cf* Folkow *et al* 1973) the design and reactivity of the resistance vessels in hypertension has been hemodynamically analyzed comparing constant flow perfusion of preferentially the hindquarter vascular beds of hypertensive (SHR) and paired normotensive control rats (NCR) Measurements of the resistance at maximal dilatation the threshold sensitivity to noradrenaline (NA) the entire dose response curve to NA and its steepness, and the maximal pressor response to supramaximal concentrations of constrictor agents revealed important differences in resistance vessel design Thus in both SHR and rats with renovascular hypertension (RHR) resistance vessel design is rapidly changed towards a luminal narrowing associated with a hypertrophic wall increase This type of structural autoregulation of the resistance vessels triggered by functional pressor influences appears to be of great importance in hypertension since resistance is then raised also when smooth muscle tone is normal

To get more insight into hypertensive vascular beds methods are needed allowing separate quantitative estimations of *e g* precapillary resistance vessels capillary exchange vessels postcapillary resistance and capacitance vessels which are specialized both in design and function For such purposes hindquarters of rats were isolated by thorough ligations of all vascular connections except the aorta and caval vein Arterial inflow pressure ($P_A$) was measured in the tail artery and venous outflow pressure ($P_V$) in a caval branch The caval vein was connected to a wide outflow tube whereby $P_V$ could be set at desired levels The preparation was connected to a constant flow perfusion pump delivering a body warm plasma substitute (oxygenated Tyrode solution with 4 % Ficoll m w about 80 000 AB Pharmacia Sweden) at known flows (Q) and levels of $P_A$ and $P_V$ The hindquarters were connected to a force displacement transducer allowing continuous precise recordings of weight shifts of the preparation From these shifts changes in intravascular fluid content and in transcapillary fluid transfer could be separately deduced (for details see *e g* Mellander 1960) In this way $P_A$ $P_V$ and Q could be set at desired levels and

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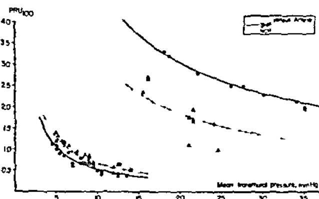



Fig 1 Pre and postcapillary resistances ($R_A$ and $R_V$) during maximal dilatation as related to mean transmural pressures $\frac{(P_A+P_C)}{2}$ and $\frac{(P_C+P_V)}{2}$. A log log linear regression of high significance was obtained for all 4 curves. The $R_A$ and $R_V$ curves of SHR and NCR differ significantly ($p < 0.001$ by a t test)

the resulting shifts in intravascular fluid volume and transcapillary fluid transfer analysed.

Maximal dilatation was first induced by papaverine and $P_A$, $P_V$ and Q were adjusted to maintain isogravimetry. Then the capillary filtration coefficient (CFC) was determined by equal rises in $P_A$ and $P_V$ and hence in $P_C$, expressing the ensuing filtration as ml/min × mm Hg × 100 g. An isogravimetric state was again established and the pre- to postcapillary resistance ratio ($R_A/R_V$) was estimated by raising first $P_A$ and second $P_V$ to such extents as to produce identical filtration rates. Then $R_A/R_V$ is largely proportional to $\Delta P_V/\Delta P_A$ because autoregulatory smooth muscle adjustments were excluded. Knowing $P_A$ and $P_V$, both $R_A/R_V$ and the isogravimetric capillary pressure ($P_{C_i}$) can be deduced (*cf* Eliassen *et al* 1973). Further knowing CFC and recording the filtration rates at different levels of $P_A$, $P_V$ and Q, $P_C$ could be calculated also for different pressure flow situations. Thus both $R_A$ and $R_V$ could be calculated also for different pressure flow situations. Thus both $R_A$ and $R_V$ decidedly higher but $R_V$ somewhat lower in SHR compared to NCR (Fig 1). However both groups showed identical values both for $P_{C_i}$ (8.5 mm Hg for the plasma substitute used) and CFC (0.065).

Then noradrenaline (NA) was infused during constant flow perfusion and initially isovolumetric conditions to produce first moderate then maximal NA constrictions, superimposing finally huge amounts of vasopressin and barium chloride to induce definitely maximal pressor responses. The constrictor responses always induced filtration indicating a $P_C$ rise in the constant flow situation due to post capillary constriction. Also during constriction CFC could be estimated fairly accurately and consequently also the $P_C$ rise by relating filtration to current CFC. Thus for each level of constriction $P_A$, $P_C$, $P_V$ and Q were known allowing calculations of both $R_A$ and $R_V$ until the resistance vessels were facing overwhelming transmural pressures. Table I gives mean arterial pressures in awake SHR and NCR together with $P_C$, $R_A$, $R_V$, $R_A/R_V$ and $P_C$ during 1) maximal vasodilatation, 2) maximal NA constriction and 3) when the maximal contractile strength was

TABLE I Mean values in 9 pairs of SHR and NCR Mean arterial pressure (BP) previously measured in the awake animals is here related to hemodynamic parameters ($P_A$ $R_A$ $P_V$ $P_C$) reflecting design and reactivity of the hindquarter vascular bed

| AWAKE B P (MM Hg) | MAX VASODILATATION | | | | | MAX CONSTRICTION (NORADRENALINE) | | | | | MAX PRESSOR RESPONSE (VASOPRESSIN + $BaCl_2$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $P_A$ * | $R_A$ * | $R_V$ * | $R_A/R_V$ | $P_{C_i}$ | $P_A$ * | $R_A$ * | $R_V$ * | $R_A/R_V$ | $P_C$ * | $P_A$ * | $R_A$ * | $R_V$ | $R_A/R_V$ * | $P_C$ |
| SHR 170 | 28 | 3 4 | 1 1 | 3 1 | 8 5 | 291 | 43 | 4 | 14 | 23 | 391 | 60 | 7 5 | 8 | 46 |
| NCR 111 | 22 | 2 5 | 1 3 | 1 9 | 8 6 | 219 | 35 | 6 | 5 8 | 34 | 258 | 40 | 8 | 5 | 44 |

estimated $R_A$ was in SHR throughout higher than in NCR largely in proportion to the raised arterial pressure in SHR while $R_V$ was somewhat lower in SHR than in NCR during dilatation and NA constriction Vasopressin and barium chloride increased $R_V$ relatively more than $R_A$ once a maximal NA response was present thus further increasing $P_C$ and filtration

Finally after administration of huge papaverine amounts to reestablish maximal vasodilatation it was possible to estimate how much tissue pressure was raised by edema as induced by $P_C$ increases during constrictions Inflow was then stopped and the pressure fall recorded where $P_V$ closely approaches zero in non edematous preparations However when tissue pressure is raised by edema $P_V$ levels off at correspondingly higher pressures As a result of bigger $P_C$ rises during NA constrictions NCR showed more edema and tissue pressure rise than SHR The reason is that NCR displayed relatively stronger $R_V$ increases to NA but in return far weaker $R_A$ increases than SHR Apparently the marked structural enhancement of $R_A$ in SHR effectively protects the subsequent vascular sections from increases transmural pressures —Summarizing the results the hypertrophic vascular changes in SHR are confined to the precapillary resistance vessels and well proportioned to the raised pressure while the capillary section is largely unchanged and the postcapillary resistance section slightly wider with somewhat weaker NA responses in SHR than in NCR

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Acta physiol scand 1974 90 657—663

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# Differentiation of the Contractile Effects of Prostaglandin $E_2$ and the C-Terminal Octapeptide of Cholecystokinin in Isolated Guinea-Pig Gallbladder

By

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## Abstract

ANDERSSON K E P HEDNER and C G A PERSSON *Differentiation of the contractile effects of prostaglandin $E_2$ and the C terminal octapeptide of cholecystokinin in isolated guinea pig gallbladder* Acta physiol scand 1974 90 657—663

In the isolated guinea pig gallbladder contractions were induced by acetylcholine (ACh) the C-terminal octapeptide of cholecystokinin (C8 CCK) prostaglandin $E_2$ ($PGE_2$) and the prostaglandin precursor arachidonic acid (ARA) The effects on these contractions induced by the inhibitor of the prostaglandin synthesis indomethacin and of the prostaglandin antagonists polyphloretin phosphate (PPP) and SC 19220 were investigated In a concentration of 0 5 μg/ml indomethacin inhibited the response to ARA but did not appreciably affect the contractions produced by ACh C8 CCK and $PGE_2$ Indomethacin 10 μg/ml reduced the responses to all the agonists probably due to a non specific depressive effect PPP 1—10 μg/ml had no effects on the contractions elicited by ACh C8 CCK and $PGE_2$ In concentrations of 100—400 μg/ml PPP caused a transient contraction in the gallbladder strips and reduced the responses to the agonists used SC 19220 2 and 8 μg/ml selectively inhibited the responses to ARA and $PGE_2$ leaving the contractions of ACh and C8 CCK virtually unaffected The inhibition of the response to $PGE_2$ was surmountable by increasing the bath concentration of the prostaglandin 5—100 time —It is concluded that local synthesis and release of prostaglandins play no essential role for the mediation of the contractile effect of C8-CCK in guinea pig gallbladder

It was previously demonstrated (Andersson *et al* 1972 1973) that the mechanical and metabolic responses of the isolated guinea pig gallbladder to the C terminal octapeptide of cholecystokinin (C8 CCK) could be mimicked by prostaglandin E (PGE) The contractions induced by C8 CCK and PGE were both preceded by a decrease in the intracellular content of cyclic AMP (cAMP) and an increase in the activity of phosphodiesterase (PDE) the cAMP inactivating enzyme Neither the contractions induced by C8 CCK nor those produced by PGE were influenced

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by adrenoceptor and cholinoceptor blocking agents or by pre treatment with tetrodotoxin suggesting that both C8 CCK and $PGE_2$ had a direct effect on the muscle cells It has been suggested that local synthesis and release of prostaglandins may be an essential link in the mediation of the response to a polypeptide hormone in the target cell (Kuehl *et al* 1970 1972 Shio *et al* 1971 Sato *et al* 1972) Because of the similarities between the mechanical and metabolic effects induced by C8 CCK and PGE in the guinea pig gallbladder we found it of interest to investigate whether prostaglandin $E_2$ is a necessary intermediate step in the development of the contractile response to C8 CCK in this tissue For this purpose arachidonic acid (ARA) the precursor of $PGE_2$ (Bergstrom *et al* 1968) and three different agents reported to inhibit the effects of prostaglandins in various tissues were used as investigational tools These agents were indomethacin a potent inhibitor of prostaglandin synthesis (Vane 1971) and the prostaglandin antagonists SC 19220 (Sanner 1969) and polyphloretin phosphate (Eakins *et al* 1970)

## Material and methods

Guinea pigs of either sex weighing 150—300 g were used The animals were killed by a blow on the head and the gallbladder was dissected out In order to obtain a strip of gallbladder tissue the fundus and cysticus parts were removed and the ring thus obtained was cut open The strip was mounted vertically in an isolated organ bath containing 25 ml Krebs solution of the following composition (mM) NaCl 118 KCl 4 6 $CaCl_2$ 2 5 $MgSO_4$ 1 15 $NaHCO_3$ 24 9 $KH_2PO_4$ 1 15 glucose 5 5 pH 7 40 The solution was maintained at 37 C and bubbled with a mixture of 95 % $O_2$ and 5 % $CO_2$ Isometric tension was recorded by means of a force transducer (Statham Ft 03) on a *Grass* polygraph The basal tension of the preparations was set at about 200 mg

Drugs The following drugs were used

The synthetic C terminal octapeptide of cholecystokinin (C8 CCK) (supplied by Dr M A Ondetti the Squibb Institute for Medical Research New Brunswick USA)

arachidonic acid (ARA) grade 1 from porcine liver (Sigma Chemical Company USA) ARA 50 mg was dissolved in 10 ml ethanol and 10 ml saline and was kept in a dark bottle at —20 C

prostaglandin $E_2$ ($PGE_2$) (Astra AB Sweden) $PGE_2$ was kept in ethanolic stock solution at – 20 C and dilutions were made with saline

polyphloretin phosphate (PPP) (Leo AB Sweden)

SC 19220 1 acetyl 2 (8-chloro-10 11-dihydrobenz b f) (1 4) oxazepine 10-carbonyl) hydrazine supplied by Dr J H Sanner Division of Biological Research G D Searle and Co Chicago USA) This substance was dissolved in ethanol to a concentration of 1 mg/ml in the stock solution and dilutions were made with saline

indomethacin Merck Sharp and Dohme supplied via Erik Lindblom AB Sweden)

acetylcholine (ACh Calbiochem USA)

The drugs were injected directly into the bath Concentrations given in the following refer to final bath concentrations

## Results

Concentrations of ACh 20 -80 ng/ml) C8 CCK (1—4 ng/ml) and $PGE_2$ (10—40 ng/ml) producing contractures of approximately the same amplitude were tested in the same gallbladder preparation The agonists were administered at intervals of about 15 min When the contractures showed good reproducibility they were taken as controls The preparation was then exposed to one of the prostaglandin inhibitors for 15—45 min After that contractions were elicited by ACh C8 CCK and $PGE_2$

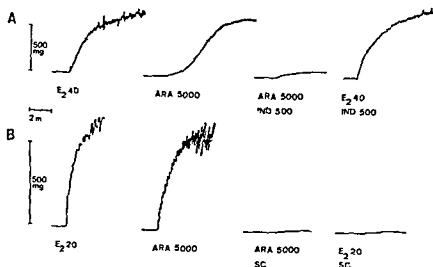



Fig 1 A B Effects of indomethacin (IND) (A) and SC 19220 (SC) (B) on contractures induced by $PGE_2$ and ARA in two isolated gallbladder strips The concentrations of the drugs are given in ng/ml The concentration of SC 19220 was 8 $\mu$g/ml

in the concentrations previously tested while the prostaglandin inhibitor was still present in the bath

*Effects of ARA* In gallbladder strips from 7 guinea pigs ARA 3—5 $\mu$g/ml produced contractions of approximately the same magnitude as PGE 20—40 ng/ml Similar to PGE ARA induced rhythmic contractile activity in most preparations

*Effects of indomethacin* Seven strips in which the response to ARA had been tested were treated with indomethacin 0 5 $\mu$g/ml for 10 min In the presence of indomethacin the amplitude of the contractions induced by ARA were reduced to less than 20 per cent of the control value (Fig 1 A) In the same strip the response to $PGE_2$ was not diminished by indomethacin in this concentration In other preparations obtained from the same 7 guinea pigs indomethacin 0 5 $\mu$g/ml was given as the first drug In these strips ARA 5 $\mu$g/ml produced effects that were less than 10 per cent of the $PGE_2$-induced contractions It was noted that the inhibitory effect of indomethacin on ARA induced contractions was difficult to abolish by repeated washing of the preparations

Ten preparations were pre treated with indomethacin 0 1—10 $\mu$g/ml for at least 30 min Indomethacin 0 1 1 and 3 $\mu$g/ml did not appreciably affect the responses to ACh C8 CCK and PGE In a concentration of 10 $\mu$g/ml indomethacin reduced the responses to all the agonists approximately to the same extent

*Effects of PPP* Ten preparations were pre treated with PPP in concentrations ranging from 1—400 $\mu$g/ml for at least 15 min In the presence of PPP 1—10 $\mu$g/ml the contractile responses to ACh C-8 CCK and $PGE_2$ were unaffected H concentrations of PPP 100—400 $\mu$g/ml produced a transient contraction

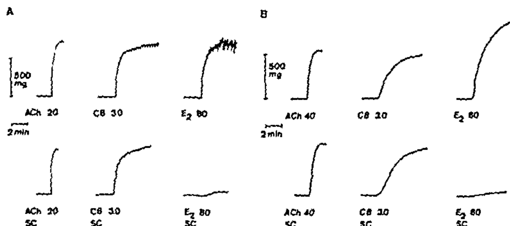



Fig 2 A B Responses of the isolated guinea pig gallbladder to ACh C8 CCK (C8) and $PGE_2$ ($E_2$) in the absence (upper part of the panel) and in the presence (lower part of the panel) of SC 19220 (SC) in the concentrations 2 μg/ml (A) and 8 μg/ml (B), respectively The concentrations of ACh C8 CCK and $PEG_2$ are given in ng/ml

gallbladder strip and often induced spontaneous activity These effects of PPP made the evaluation of its possible antagonistic effects difficult However the contractile responses to all the agonists were reduced approximately to the same degree in the presence of PPP 100—400 μg/ml

*Effects of SC 19220* SC 19220 in concentrations between 2 and 25 μg/ml was left in contact with 42 preparations for at least 15 min In the presence of SC 19220 2 μg/ml the responses to ACh and C8 CCK were not appreciably influenced (Fig 2 A) On the other hand the contractions induced by PGE were reduced by 71 per cent in the mean (range 30—100 per cent n = 9)

When the concentration of SC 19220 was increased to 8 μg/ml, the response to PGE was markedly diminished (Fig 2 B) In 14 preparations the contractile amplitude was reduced by 95 per cent in the mean (range 77—100 per cent) The contracting effects of ARA 5—12 5 μg/ml were almost completely inhibited by SC 19220 in this concentration (Fig 1 B) The contractions induced by ACh and C8 CCK were little influenced Usually a slight reduction in contractile tension was observed but in a few preparations there was an initial increase in contractile amplitude In the presence of SC 19220 8 μg/ml the mean change in tension induced by ACh was −15 per cent (range −50—+15 per cent n = 9) and by C8 CCK −15 per cent (range −50—+10 n = 9) compared to controls

In a few experiments the effects of SC 19220 in a high concentration 25 μg/ml were investigated In this concentration the antagonist completely abolished the responses to PGE but also consistently reduced the amplitudes of ACh and C8 CCK induced contractions Sanner (1972) reported 25 μg/ml of SC 19220 to approximate the maximum concentration obtainable in Ringer solution because of the limited solubility of the agent

By increasing the concentration of PGE 5—100 times the contractile response

to this agent could be restored to control values in the presence of SC 19220 in a concentration of 8 $\mu$g/ml but not in a concentration of 25 $\mu$g ml The inhibitory effects of SC 19220 were reversible and 15 min after the substance was washed out from the bathing fluid the contractile response to $PGE_2$ in the concentration initially tested was restored.

In order to further investigate the effects of SC 19220 on the contractions induced by ACh, C8-CCK, and $PGE_2$ each of these substances was added to the bathing fluid in cumulatively increasing concentrations in the absence and the presence of the inhibitor However these experiments were complicated by the finding that longlasting exposure to $PGE_2$ induced spontaneous rhythmic activity in many of the preparations This was also seen after addition of C8-CCK but less pronounced. It was observed that when repeated control series were performed with $PGE_2$ the logdose response curve was variably displaced to the left suggesting an increasing sensitivity to the substance during the course of the experiment.

Despite this it could be demonstrated that the cumulative logdose response curve for $PGE_2$ in most gallbladder strips was displaced to the right in the presence of SC 19220 in concentrations of 2 and 8 $\mu$g/ml. Corresponding curves for ACh and C8-CCK were not significantly changed. When SC 19220 was added in a concentration of 25 $\mu$g/ml the maximum responses to ACh C8-CCK, and $PGE_2$ were all depressed and this effect was not surmountable by increasing the concentrations of the agonists in the bathing fluid.

## Discussion

Prostaglandins are known to be present in most tissues in the body including the gastrointestinal tract (Bergström *et al* 1968 Bennett and Fleshler 1970 Wilson 1972) but their physiological role is not known. The effects of endogenous prostaglandins are belived to be brought about preferentially by increased local synthesis in the tissue concerned (Ramwell and Shaw 1970) The contractile response to ARA in the isolated guinea pig gallbladder demonstrated in the present study suggests that synthesis of prostaglandins can take place also in this tissue

The effects of agents supposed to act through an intermediate step involving prostaglandins should be counteracted by substances that inhibit prostaglandin synthesis. Indomethacin in concentrations of 1—4 $\mu$g ml has been shown to inhibit the synthesis of prostaglandins in various tissues (see *e g* Vane 1971 Ferreira *et al* 1971 Vane and Williams 1972) In the present experiments on isolated gallbladder indomethacin 0.5 $\mu$g/ml almost completely inhibited the contractile response to ARA without affecting the response to exogenously added $PGE_2$ This suggests that indomethacin in this concentration effectively reduced the prostaglandin synthesis in the gallbladder strips and that ARA by itself did not have a prostaglandin like effect. However there was no reduction of the contractile response of the gallbladder preparations to C8-CCK or exogenously added $PGE_2$ despite the presence of indomethacin in concentrations up to 5 $\mu$g ml in the bathing fluid.

The obtained results suggest that impaired synthesis of prostaglandins does not seriously affect the ability of the gallbladder strips to respond to C8 CCK Indomethacin 10 $\mu$g/ml prevented part of the C8 CCK induced response but this seemed to be attributable to a non specific depressive effect of the drug as the contractile effects induced by ACh and $PGE_2$ were similarly affected This depressive effect by indomethacin may be explained by its ability to inhibit the influx of calcium into the muscle cell (Northover 1971)

The agents at present in use for antagonizing effects of exogenously added prostaglandins may be considered only as prototypes for the development of more specific and potent compounds (Sanner 1972) However, several investigations of the effects of the antagonists PPP and SC 19220 (see e g Sanner 1969 Eakins *et al* 1970 Bennett and Posner 1971) have defined their drawbacks and shown that they may be useful pharmacological tools

As the present experiments show PPP in concentrations (1—10 $\mu$g/ml) having a selective prostaglandin antagonistic effect in other smooth muscle preparations such as jird colon and rabbit jejunum and uterus (Eakins *et al* 1970) seemed to be without this action in the guinea pig gallbladder The evaluation of the antagonistic effect of PPP in high concentrations (100—400 $\mu$g/ml) was disturbed by the contractile actions of PPP itself but there was no evidence of a selective prostaglandin antagonistic effect Transient contractile effects of PPP have been observed by other investigators (Eakins *et al* 1970)

By means of SC 19220 it was possible to counteract the effects of PGE and ARA leaving the contractions induced by C8 CCK and acetylcholine virtually unaffected The blocking effect of SC 19220 2 and 8 $\mu$g/ml was surmountable by increasing the concentration of PGE suggesting a competitive manner of inhibition In experiments with cumulative dose response curves it was also seen that the PGE curve was shifted to the right in the presence of SC 19220 During the course of the experiments with cumulatively increased prostaglandin concentrations in the bath it was observed that the sensitivity to $PGE_2$ markedly increased in many preparations This spontaneous change could not readily be corrected by running control tests and it invalidated the determination of PA values for the blocking activity of SC 19220 The consistent and selective inhibitory effect of SC 19220 on PGE induced submaximum contractions of the gallbladder strips does not favour the idea that PGE plays an important role in the mediation of the contractile effect of C8 CCK in this tissue This is consonant with the previous observation that a gallbladder strip maximally contracted by PGE is able to further increase its tension on the addition of C8 CCK in a low concentration (Andersson *et al* 1973)

Kuehl *et al* (1970 1972) produced evidence that prostaglandins constitute a necessary intermediate step in the LH induced activation of adenyl cyclase in mouse ovary and the findings of Sato *et al* (1972) suggested that prostaglandins are of similar importance for TSH stimulation of cyclic AMP formation in the thyroid gland Shio *et al* ([illegible]) discussed the possibility that local synthesis and release of

prostaglandins mediate the effects of polypeptide hormones in the target organ PGE has a conspicuous similarity to C8 CCK concerning effects on tension cyclic AMP level and PDE activity in gallbladder muscle (Andersson *et al* 1972 b 1973) and it was tempting to speculate whether the effects of C8 CCK were mediated by PGE However the present results show that it is not possible to block the contractile effects of C8 CCK either by the inhibitor of prostaglandin synthesis indomethacin or by the prostaglandin antagonist SC 19220 which effectively blocks the response to PGE These findings suggest that PGE is not essential in the mediation of the contractile effects of C8 CCK in gallbladder muscle

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Acta physiol scand 1974 90 664—672

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# The Effects of Physical Exercise on Fat Cell Metabolism in the Rat

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## Abstract

KRAL J G B JACOBSSON U SMITH and P BJORNTORP *The effects of physical exercise on fat cell metabolism in the rat* Acta physiol scand 1974 90 664—672

Rats trained by repeated swimming bouts were compared with rats swimming once and with sedentary controls Training caused a decrease in body weight, body triglyceride and fat cell size Fasting plasma insulin was also decreased However plasma free fatty acids and glycerol were not changed Incorporation of labelled glucose into fat cell triglycerides was decreased by physical training which also caused a disappearance in the fat cell size dependence of adipose tissue glucose metabolism The response to insulin of this pathway in enlarged fat cells was increased by physical training After a single exhaustive swim the cell size dependence of triglyceride labelling of adipose tissue apparently also disappeared It was suggested that physical training leads to an adaptation of adipose tissue metabolism with an increased insulin sensitivity This effect as well as the disappearance of the cell size dependence of adipose tissue metabolism might hypothetically be due to a diminished glucose flow to adipose tissue caused by exercise

Physical training is followed by a weight decrease in the rat (Mayer *et al* 1954 Stevenson *et al* 1966 Crews *et al* 1969 Oscai and Holloszy 1969) as well as in man Pařízková 1963 Skinner *et al* 1964 Pollock *et al* 1971 Bjorntorp *et al* 1972 Bjorntorp *et al* 1972) due to a decrease in body fat This implies that training alters adipose tissue metabolism towards increased lipid mobilization and/or decreased lipid assimilation Studies on plasma free fatty acid (FFA) concentration after physical training Johnson *et al* 1969 Froberg *et al* 1972) do not necessarily reflect adipose tissue metabolism because the fractional turnover rate of FFA may well be altered due to an adaptation of the enzyme capacity for aerobic oxidation after physical training Holloszy 1967) An increased uptake of FFA has thus been demonstrated in the perfused leg after physical training (Keul *et al* 1970)

Direct studies of adipose tissue metabolism in connection with physical training are scarce Recently Askew *et al* 1973) and Froberg *et al* (1972) found an in

creased spontaneous and noradrenaline stimulated lipolysis when it was related to adipose tissue wet weight in trained rats This, however may well be due to the weight-decreasing effect of physical training which leads to an increased number of fat cells per unit wet weight in the adipose tissue of trained rats simply because of the diminution of fat cell weight When related to the amount of DNA these differences disappeared (Froberg *et al* 1972) though the fact that the main part of the DNA in the epididymal fat pad resides in other cells than fat cells (Rodbell 1964) makes these results difficult to interpret

The substantial effect of physical training on adipose tissue mass and the lack of knowledge of its effects on fat cell metabolism made it of interest to study this question systematically Thus in the present study rats were trained physically by swimming and the effects of this procedure on body composition plasma metabolites and adipose tissue metabolism were studied The effect of a single bout of exercise was also examined

## Experimental procedures

Male Sprague Dawley rats weighing 300—350 g were fed commercial rat pellets *ad libitum* (E s Sodertalje) containing by weight 5 % fat 55 % carbohydrate and 22 5 % protein plus minerals and vitamins They were housed in individual cages and given tap water freely One group (n = 9) was exercised by daily swimming one group (n = 7) swam only once and one group (n = 8) served as a control

The swimming was performed in a tank divided into four compartments allowing four rats at a time to swim individually The rats could not rest on the bottom or the walls The water was 29—32 C and contained a detergent to diminish the floating capacity of the fur A weight (2.5 g per 50 g b wt.) was attached to the tail The rats swam to exhaustion defined as the time when they were no longer able to return to the surface to snatch air The time for each swimming session was about 10 min The trained group swam twice daily 5 days a week and once daily the remaining two days each week for 6 weeks The last swim was 24 h before sacrifice in both the trained and single bout group The controls were kept in the same manner as the exercised animals After an overnight, 12 h fast with access to water the animals were anesthetized by intraperitoneal administration of 50 mg pentobarbital (Mebumal ACO Sweden) per kg body weight. At a well-defined level of anesthesia with muscle relaxation and normal breathing the animals were sacrificed by carotid exsanguination The first portion of blood was taken for the analyses

*Blood carcass and muscle analyses*

Blood was collected in a heparinized tube for the determination of glucose Levin and Linde 1962 glycerol (Laurell and Tibbling 1966) plasma insulin (Hales and Randle 1963 triglycerides (Carlson 1959) cholesterol (Cramer and Isaksson 1959) and FFA (Trout *et al* 1960)

Adipose tissue was removed from defined sites in the perirenal proximal and distal parts of the epididymal fat pad for fat cell size determination (Sjostrom *et al* 1971) The contralateral epididymal fat pad was removed *in toto* and weighed The proximal part of the pad was incubated for the determination of glucose incorporation into triglycerides

The remaining carcass was ground in a meat grinder lyophilized and the remnants homogenized to a powder in a Waring blendor Lipids were extracted from a powder aliquot for the determination of total triglyceride content. The homogeneity of the powder was ascertained by extractions of several powder aliquots the results showing no larger difference than the method for triglyceride determination. Repeated extraction of the once extracted powder yielded insignificant additional triglyceride Addition of the triglyceride content of the removed fat tissue yielded total body triglyceride Succinic oxidase activity in the vastus lateralis muscle was determined (Bjorntorp *et al* 1970) in another series of identically trained rats.

After determination of fat cell size an average cell weight was calculated assuming a triglyceride density of 0 91 Fat cell number was then estimated by dividing adipose tissue triglycerid weight by mean fat cell weight

TABLE I Body composition of rats after a single swim or repeated bouts of swimming exercise (= trained) and of controls

| Group | n | Body weight (g) | Total body triglyceride (g) | Epididymal fat pad wet weight (g) |
|---|---|---|---|---|
| Controls | 7 | 412±4 | 32 5 57 3 30 0++ average 39 9 | 6 5±0 5 |
| Trained | 8 | 353±4*** | 16 6 18 0 30 1++ average 21 6 | 4 3±0 3** |
| Single | 7 | 404±4 | — | 6 3±0 2 |

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

Comparisons with controls Means±S E
Results from one rat missing
Only 3 rats analysed

*Incorporation of labelled glucose into adipose tissue triglycerides*
The procedure used has been described in detail previously (Smith 1970) Briefly fragments from the tissue samples (total weight 300—500 mg) were incubated in medium 199 (Statens Bakteriologiska Laboratorium Stockholm Sweden) modified to a glucose concentration of 1 0 mM After addition of 0 15 μC of [$^{14}$C-1] glucose (New England Nuclear Corporation Frankfurt am Main West Germany) per 2 ml of medium the incubations were performed with or without the addition of 1000 μU insulin (glucagon free porcine insulin Vitrum AB Stockholm Sweden) for 2 h at 37 C and at pH 7 4

The tissue lipids were extracted with chloroform methanol 2 1 (v/v) (Folch *et al* 1957) Aliquots of the chloroform phase were evaporated to dryness and 10 ml scintillation fluid added The radioactivity was determined in a Packard Tri Carb liquid scintillation spectrometer (Packard La Grange Illinois USA) Quenching was corrected for by means of internal standardization

Aliquots of the chloroform phase were also taken for the determination of the triglyceride content (Carlson 1959)

*Calculations*
The metabolic activity of the adipose tissue is expressed in terms of the cellularity of the tissue fragments Fat cell surface was calculated for each cell from its diameter determined according to Sjöstrom *et al* (1971) and the surfaces so obtained were averaged

For statistical analysis Student's t test and the sign test were utilized (Snedecor and Cochran 1967

## Results

*Body composition plasma variables and succinic oxidase activity in muscle*
The trained rats were lighter and contained less triglyceride (Table I) than controls. Fat cell size was significantly diminished in the epididymal fat pads but fat cell number was not changed Plasma lipids glycerol and blood glucose did not differ from the controls while plasma insulin was significantly lower (Table II) A single swim had no noticeable effect on these variables (Table I and II) The fact that the training procedure was effective is illustrated by the fact that succinic

| Fat cell weight: Proximal epididymal fat pad (µg) | Distal epididymal fat pad (µg) | Perirenal fat pad (µg) | Epididymal fat cell number (x10 ) |
|---|---|---|---|
| 0.58±0.05 | 0.34±0.05 | 0.38±0.05 | 10.1±0.9 |
| 0.36±0.02** | 0.21±0.02* | 0.37±0.06 | 9.8±1.0 |
| 0.54±0.04 | 0.28±0.02 | 0.37±0.06 | 11.2±0.9 |

TABLE II Blood and plasma variables of rats after a single swim or repeated swimming exercise (trained) and of controls

| Group | n | Glucose (mg/100 ml) | Insulin (µU/ml) | Glycerol (mM) | Free fatty acids (µM) | Triglycerides (mM) | Cholesterol (mg/100 ml) |
|---|---|---|---|---|---|---|---|
| Controls | 7+ | 70±3 | 17±2 | 0.10±0.01 | 469±53 | 0.32±0.06 | 80±6 |
| Trained | 8 | 70±4 | 12±1* | 0.10±0.01 | 500±34 | 0.38±0.05 | 74±3 |
| Single swim | 7 | 65±5 | 18±2 | 0.10±0.01 | 550±30 | 0.30±0.04 | 84±6 |

* $p < 0.05$ Comparison with controls Means±S E
+ Results from one rat missing

TABLE III Mean rate of glucose incorporation and increase over basal level

| | Basal glucose incorporation (nmoles/10 cells) | Difference with added insulin (nmoles/10 cells) | Per cent increase over basal incorporation | p-level |
|---|---|---|---|---|
| Controls | | | | |
| One swim | 27.9±3.9 | +19.3±2.0 | 60 | < 0.01 |
| Six weeks of swimming | 27.1±2.2 | +19.0±4.9 | 60 | < 0.01 |
| | 16.5±1.2 | +17.7±3.2 | 110 | < 0.01 |

* $p < 0.05$ Comparisons with controls
+ The limits of significance were determined with sign test
Means±S E

oxidase activity in the muscles from similarly trained rats was significantly increased (22.2±1.9 and 31.3±2.1 µl O /mg protein×h for controls n = 9 and trained rats n = 11 respectively Means±S E $p < 0.01$)

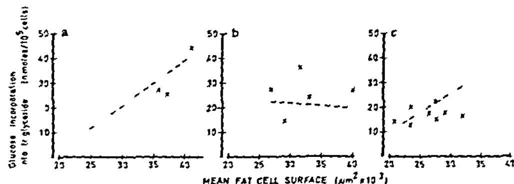



Fig 1 Effect of cell size on the incorporation of labelled glucose into the triglyceride of a controls rats ($r = 0.92$ $p < 0.01$) and b rats swimming once ($r = 0.24$ $p > 0.1$) or c trained for 6 weeks ($r = 0.37$ $p > 0.1$)

*Incorporation of labelled glucose into adipose tissue triglycerides*

There was no difference in the average rate of glucose incorporation between the control rats and those subjected to physical exercise once However the incorporation was significantly decreased in the group of rats swimming for six weeks (Table III

The incorporation of labelled glucose into the triglycerides was also studied as a function of mean fat cell size In the control group a significant positive correlation was found between the incorporation of glucose and mean fat cell surface (Fig 1 a $r = 0.92$ $p < 0.01$ However no correlations were found between these parameters in the rats swimming once (Fig 1 b $r = 0.24$ $p > 0.1$) or for six weeks (Fig 1 c $r = 0.37$ $p > 0.1$)

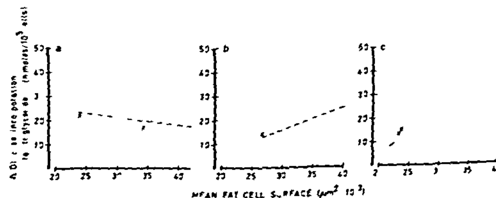



Fig 2 The difference between basal and insulin stimulated glucose incorporation into triglyceride as a function of fat cell surface a controls $r = -0.3$[illegible] $p > 0.01$ b after a single swim $r = 0.31$ $p > 0.1$ c after 6 weeks of daily training $r = 0$ [illegible] $p < 0.05$

Added insulin had a significant effect in all groups (Table III). However, the stimulating effect of insulin on a percentage basis was more pronounced in the rats swimming for six weeks (Table III). For both the controls and the rats swimming once the average increase over basal incorporation was 60 per cent whereas for the trained rats it was 110 per cent. Further analysis of the absolute effect of insulin on glucose incorporation related to the mean fat cell surface is shown in Fig. 2. The coefficient of regression increased with increasing amount of work being higher in the rats swimming once and still higher in repeatedly swimming rats indicating that the insulin responsiveness increases with work mainly in the larger fat cells.

## Discussion

Physical training causes a decrease in body weight at least partly attributable to a reduced body triglyceride content and this is in turn associated with a diminished fat cell size. These effects of physical training have been observed before (Mayer et al. 1954, Stevenson et al. 1966, Crews et al. 1969, Oscai and Holloszy 1969). Blood glucose and plasma lipids were not changed while the plasma insulin level was significantly lower. No significant effects on any of these variables were registered the day after a single bout of exercise.

The number of the epididymal fat cells was not changed by physical training but the size of these cells diminished significantly leading to a 35 % reduction in pad weight. Plasma glycerol and FFA concentration were not changed by physical training. It has previously been shown that there is a correlation between cell size and lipolysis *in vitro* (Faulhaber et al. 1969, Smith 1970, Goldrick and McLoughlin 1970, Björntorp and Sjöström 1972). The fact that the glycerol and FFA levels were not changed in spite of the reduced adipose mass because of smaller fat cells may indicate enhanced lipolysis in these smaller fat cells as suggested by other authors (Fröberg et al. 1972, Askew et al. 1973). The decrease in basal glucose incorporation into triglyceride suggests another reason for the decrease in fat cell size namely decreased accumulation of fat. Which of these alternatives is the more important is the subject of further studies in our laboratory.

It is of interest that some of the findings after six weeks of training were also found after a single bout of exercise. The dependence on fat cell size of basal glucose incorporation into triglyceride was thus removed not only by physical training but also by a single work load. Furthermore there was a trend towards an increased insulin sensitivity in the larger fat cells after a single work session in spite of the fact that there was no measurable change in fat cell size.

The similarities between the effects exerted by training on the one hand and a single work session on the other on adipose tissue metabolism seem to be explicable in one of at least two ways. The first is that the effect on glucose incorporation in adipose tissue is a result of a single bout of exercise. This phenomenon is seen as a result of the single swim as well as the last swim in the trained repeatedly exercised rats. With this alternative the adipose tissue changes should be temporary

lasting for some limited time period after the last training session The other possibility is that physical work not only causes these immediate effects but after repeated work also more long lasting adaptive effects on the metabolism in analogy with those seen in muscle after physical training (Holloszy 1967) The present work does not reveal which of these two alternatives may be the correct one or whether a combination of both of these effects is actually responsible for the findings in the trained rats To solve this question studies of adipose tissue metabolism at different times after a single exercise bout are needed to investigate the duration of the effects

The positive correlation between adipose cell size and metabolism repeatedly reported previously (Björntorp 1966 Zinder *et al* 1967 Björntorp and Karlsson 1970 Goldrick and McLoughlin 1970 Smith 1971) is dependent upon the carbohydrate content of the diet because this correlation may be increased at least for pathways utilizing glucose as the main substrate during a period of high carbohydrate feeding This is true for both glucose label incorporation into triglycerides and carbon dioxide (Sjöström *et al* in preparation) as well as *de novo* fatty acid synthesis (Sjostrom 1973) Furthermore rats on a carbohydrate rich diet show such a dependence while it is lacking in rats fed a fat rich diet (Smith *et al* 1974) Recent findings in fat cell cultures that the metabolic activity in short term studies subsequent to culture is profoundly influenced by the insulin and glucose levels during the culture period (Smith 1974) may be of importance to this qustion This may indicate that a certain minimum exposure of adipose tissue to glucose and/or insulin is necessary to develop the size-dependence of the glucose metabolism of fat cells The lack of dependence in trained rats might then indicate that such an exposure has diminished indicating that glucose flow to adipose tissue is diminished by physical training This in turn would produce diminution of the fat cells

Previous reports have shown the dependence of insulin sensitivity on the size of fat cells Smith 1970 Salans and Dougherty 1971) The nutritional state influences the insulin response of the metabolic pathways of glucose (Salans and Dougherty 1971) and diet content of carbohydrate markedly modifies the insulin sensitivity of the pathways of both triglyceride synthesis and breakdown (Smith *et al* 1974) The present work indicates that insulin sensitivity of fat cells is dependent on physical activity as well

It is then clear that the insulin sensitivity of adipose tissue is dependent not only on fat cell size but also on several other factors

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# A Pharmacological Study of Intestinal Vasodilator Mechanisms in the Cat

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## Abstract

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The intestinal vasodilator responses evoked by close i a administration of cholecystokinin (CCK) and secretin mechanical stimulation of the mucosa and transmural electrical field stimulation were abolished by the 5 hydroxytryptamine (5 HT) antagonist dihydroergotamine given in doses sufficient to block the vascular effects of i a injected 5 HT Corresponding results were obtained by making the cat small intestine tachyphylactic to 5 HT Nervous blockade (tetrodotoxin) inhibiting the intestinal blood flow increase after mechanical mucosal or electrical field stimulation left the vascular responses to exogenous CCK and secretin unaffected An increased 5 HT content in venous blood from the intestine was demonstrated during vasodilatations caused by electrical field stimulation A participation of intestinal 5 HT possibly as a transmitter substance in the vasodilator mechanism evoked by mechanical mucosal stimulation or electrical transmural field stimulation is suggested.

Mesenteric blood flow increases following instillation of fat or acid into the duodenum due at least in part to the release and action of the duodenal hormones cholecystokinin (CCK) and secretin (Fara Rubinstein and Sonnenschein 1972) Further mechanical stimulation of the intestinal mucosa causes an intestinal vasodilator response suggested to be mediated through a local intestinal nervous reflex (Biber Lundgren and Svanvik 1971) Also nervous structures in the intestinal wall can be directly activated by transmural electrical field stimulation (Biber Fara and Lundgren 1973 a) causing an intestinal vasodilatation Thus it seems likely that different trigger mechanisms are involved in the local regulation of intestinal blood flow

The question of whether a common mediator mechanism might be involved in the above mentioned intestinal vascular responses has been raised by several recent studies involving 5 hydroxytryptamine (5 HT) and tetrodotoxin The normal intestinal wall contains abundant amounts of 5 HT (Vialli 1966) and local i a administration of 5 HT evokes an intestinal vasodilator response which regarding its effects on the consecutive vascular sections closely resembles that induced

secretin CCK electrical field stimulation and mechanical mucosal stimulation (Biber Fara and Lundgren 1973 b c) Additionally the intestinal vasodilatation evoked by mechanical mucosal stimulation is abolished by 5 HT receptor blocking agents and by tetrodotoxin (Biber *et al* 1971) Thus the present experiments were performed to investigate whether 5 HT is acting as a transmitter substance in an intramural nervous vasodilator reflex in the intestine and is thereby mediating the hyperemic responses to hormonal electrical and mechanical stimulation

## Methods

*A Operative and recording procedures* Experiments were performed on 26 cats of both sexes weighing 2 0 3 6 kg The cats were deprived of food for 24 h and anesthetized with chloralose i v (50—60 mg/kg b w ) after ether induction

The operative procedures were similar to those of earlier studies (for details see Biber *et al* 1971) A proximal jejunal segment of 15—35 g was isolated but left *in situ* while the rest of the intestine was removed Venous outflow was recorded by an optical drop recorder unit operating an ordinate writer All splanchnic nerves were cut and the nerve trunks along the superior mesenteric artery sectioned and placed in ring electrodes for efferent stimulation in some experiments Arterial blood pressure was monitored from a femoral artery *via* a Statham pressure transducer (P23AC) pressure and flow being recorded on a Grass polygraph Local i a injections and infusions were given by means of a catheter in a small branch of the superior mesenteric artery

*Via* a soft plastic cannula placed in the intestinal lumen motility could if desired be continuously measured by a Statham low pressure transducer (P23BC) both ends of the segment then being tied off

*B Mechanical stimulation* The intestinal mucosa was mechanically stimulated by continuously pulling a [illegible]—3 cm long plastic tube with an outer diameter of 5 mm back and forth along the lumen by means of soft strings tied to its ends (for details see Biber *et al* 1971)

*C Transmural electrical field stimulation* To perform a transmural electrical field stimulation a soft plastic tube (outer diameter 2 mm) with a flattened silver wire wrapped along its length was cautiously placed inside the intestinal lumen as the cathode A flexible silver wire closely enwrapping the jejunal preparation served as the outer anodal electrode The *in situ* jejunal segment was placed in a lucite chamber containing saline at 38 C (For details see Biber *et al* 1973 a) Electrical stimulation was delivered with constant current at a strength of 60 100 mA a frequency of 60 80 Hz and a pulse duration of 8 10 ms stimulation parameters which elicited an optimal vasodilator response

*D Determination of 5 HT in plasma* During control and field stimulation period arterial and venous blood samples (volume 3 4 ml) were concomitantly collected *on ice* from a femoral artery and the mesenteric vein The samples were centrifugated at 0 C and 0 05 ml of [illegible] acid and 0 05 ml of a 10 % EDTA solution were added to the plasma, after which they were kept frozen at − [illegible]0 C and usually analysed within 24 h For biochemical determination of the 5 HT plasma concentration the method of Anden and Magnusson [illegible] was used Release or uptake of 5 HT from the gut was calculated from the arteriovenous concentration differences and the recorded total intestinal blood flow

*E Drugs and hormones* The following drugs were used dihydroergotamine (Ostan [illegible]® Sandoz) 5 hydroxytryptamine creatinine sulphate (May and Baker) atropine sulfate papaverine hydrochloride and tetrodotoxin (Calbiochem) The hormones cholecystokinin (CCK) and secretin were obtained from the Gastrointestinal Research Unit Karolinska Institutet Stockholm The cats were routinely given atropine i v (1 mg/kg b w ) at the start of the experiment

## Results

*A The effect of a pharmacological 5 HT blockade* In 9 expts a 5 HT antagonist (dihydroergotamine DHE) was studied concerning its effect on the vasodilatations induced by secretin CCK mechanical mucosal and electrical field stimulation

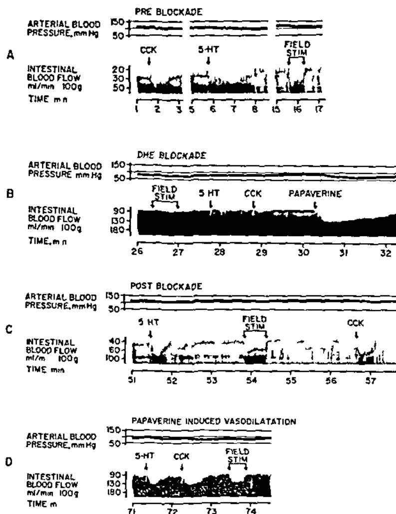



Fig 1 The effects of a 5 HT receptor blockade (dihydroergotamine DHE 1 $\mu$g/kg b w i a.) on the intestinal vasodilator responses evoked by transmural electrical field stimulation and by i.a. injection of cholecystokinin (CCK 1 25 Ivy dog U) and 5-HT (10 $\mu$g) None of these experimental procedures induced any vasodilatation immediately after DHE administration (panel B) while control effects (panel A) could be induced 40 min after DHE injection (panel C) As control is also shown that papaverine (0 25 mg i a) can induce a vasodilatation during the DHE blockade (panel B) Furthermore panel D illustrates that 5 HT CCK and field stimulation causes intestinal vasodilatations during a papaverine induced hyperemia of the same magnitude as that caused by DHE (panel B)

DHE (1 mg/kg b w administered locally i a ) blocked completely the vasodilator effect of 10 $\mu$g i a injected 5 HT DHE which also has $\alpha$ adrenergic receptor blocking properties did not in these doses fully inhibit the adrenergic vasoconstrictor response to sympathetic nerve stimulation During such a pharmacological 5 HT blockade the intestinal vessels did not at all respond to either i a administered

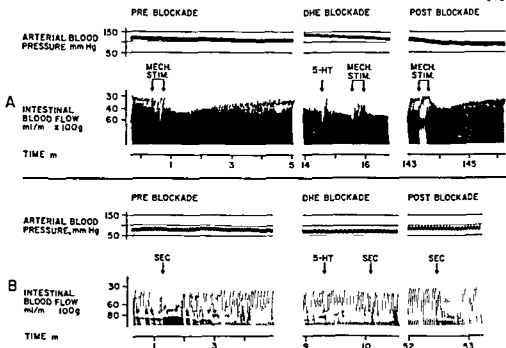



Fig 2 The effects of 5 HT blockade (dihydroergotamine DHE 1 mg/kg b w ) on the intestinal vasodilator responses to mechanical stimulation (panel A) and to i a. administered secretin (1 25 Ivy dog U panel B) During blockade the intestinal vasculature is irresponsive to mechanical mucosal stimulation and i a secretin the effectivness of the blockade beeing demonstrated by i a injections of 5 HT (10 $\mu$g) The i a. injected volumes induce a small transient artefact in the blood flow registrations

secretin or CCK (1 0—2 5 Ivy dog U) or to mechanical and electrical field stimulation but markedly so to i a papaverine (Fig 1 and 2) Within 40 min after the DHE injection the vasodilator response to all the above stimuli and to 5 HT returned to control (Fig 1 and 2)

The DHE administration *per se* usually induced a moderate intestinal vasodilatation during 5 HT blocking periods This could however not explain the absence of the vasodilator responses to the studied stimuli since these were unaffected when a corresponding degree of vasodilatation was maintained by i a papaverine infusion (Fig 1 D)

Control experiment showed that the solution in which DHE was dissolved had no effect of its own on the studied dilator mechanisms

*B The effect of intestinal tachyphylaxis to 5 HT* Tachyphylaxis to 5 HT has been demonstrated in most smooth muscle preparations *in vivo* as well as *in vitro* (Erspamer 1966 b) Such a tachyphylaxis could also be induced in the intestinal vascular bed by a continuous i a administration of 5 HT in increasing amounts (from 10 $\mu$g/min up to 200 $\mu$g/min) Within 10 min this procedure performed in

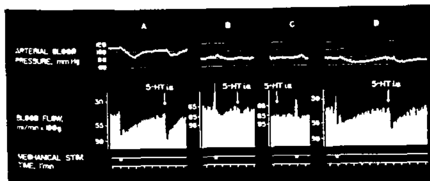



Fig 3 The effect of 5 HT tachyphylaxis in the small intestine induced by continuous i a. 5 HT infusion in increasing doses on the intestinal blood flow increase seen after mechanical mucosal stimulation (signal) Panel A illustrates the results obtained during control conditions Panel B and C shows the effects of a partial and total tachyphylaxis respectively Panel D illustrates the return to control conditions within 30 min after total tachyphylaxis Th vascular 5 HT tachyphylaxis was tested by giving i a injections of 5 HT (10 μg)

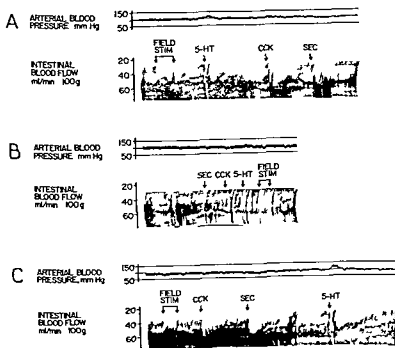



Fig 4 The effects of an intestinal 5-HT tachyphylaxis induced by continuous a. infusion of 5-HT on the intestinal vasodilator responses to a secretin and cholecystokinin (CCK 1 25 Ivy dog U) and to transmural electrical field stimulation Panel A control panel during total tachyphylaxis panel C 30 min after total tachyphylaxis 5 HT (10 μg) was injected i a. as a control of the degree of tachyphylaxis

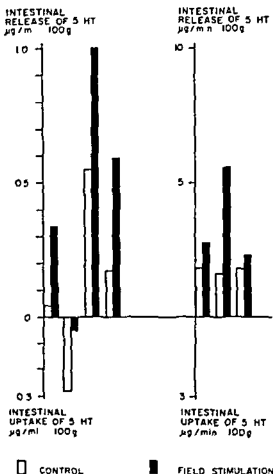



Fig. 5. The effect of a transmural electrical field stimulation on the intestinal 5 HT release into venous blood. In one experiment an intestinal uptake of 5 HT was recorded. For details see text.

6 cats led to an intestinal vascular unresponsiveness to further 5 HT administration. During the tachyphylaxis periods blood flow either remained unchanged (3 expts.) or was slightly increased (3 expts.) beyond control but the intestinal vasodilatation could readily be induced by i.a. papaverine infusions. However, throughout the period of tachyphylaxis the vasodilator responses to secretin, CCK, mechanical mucosal and electrical field stimulation were completely abolished (Fig. 3 C and 4 B). Return to control conditions occurred 30—60 min after the tachyphylaxis period. Apart from a transient motility increase at the start of the 5 HT infusion intestinal motility remained largely unchanged during the periods of tachyphylaxis.

*C. The effect of nervous blockade by tetrodotoxin.* Tetrodotoxin selectively blocks nervous conductivity without impairing smooth muscle function *per se* (Narahashi *et al.* 1964, Nonomura *et al.* 1966, Gershon 1967, Ozawa and Sugawara 1967). In 4 expts. tetrodotoxin (2—4 mg/kg b.w.) was given close i.a. to the intestine. This caused an immediate local nervous blockade as demonstrated by the total block of

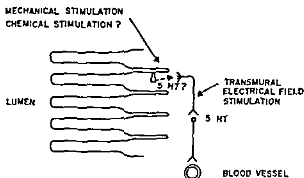



Fig 6 The hypothetical arrangement of the local nervous reflex inducing an intestinal vasodilatation after mechanical stimulation of the intestinal mucosa and upon transmural electrical field stimulation The number of neurons in the nervous arch is arbitrarily set to 2

sympathetic vasoconstrictor responses lasting up to 15—30 min with a subsequent gradual return of nervous reactivity During this nervous blockade the intestinal vasodilator response to 5 HT (10 μg), administered close i a was either blocked or reversed into a vasoconstriction as reported earlier (Biber *et al* 1973 b) However the vasodilator response to secretin or CCK (1 0—2 5 U i a ) was not influenced although repeated periods of nervous blockade were induced in all experiments of this series

*D The release of 5 HT from the small intestine during field stimulation* To obtain evidence for the possible role of 5 HT as a transmitter substance in the gut wall 5 HT concentrations in the intestinal venous plasma were determined in 6 cats before and during a period of intestinal vasodilatation induced by transmural electrical field stimulation The results summarized in Fig 5 show that the intestinal release of 5 HT is markedly increased during this type of hyperemia the relative increase in 5 HT release from the intestine amounting to 2ು3 ± 91 per cent (mean ± S E n = 7) In all the experiments of Fig 5 the arterial 5 HT plasma concentration remained fairly unchanged during field stimulation Hyperemia of corresponding magnitude produced by local i a infusion of papaverine was associated with a reduced 5 HT release as compared to control

## Discussion

In the present investigation on cats the effects of 5 HT blockade were studied with respect to several vasodilator mechanisms that have been proposed to be involved in the physiological control of intestinal blood flow These include the action of secretin and cholecystokinin (CCK) normally released in physiological amounts by amino acids and fat in the duodenum (Fara *et al* 1972) mechanical stimulation of the mucosa (Biber *et al* 1971) as well as transmural electrical field stimulation (Biber *et al* 1973 a) The vasodilatation evoked by mechanical mucosal stimulation was earlier demonstrated to be abolished by blocking the intestinal vasodilator effects of 5 HT with bromo LSD (Biber *et al* 1971) To elucidate the extent t which the responses to other vasodilator stimuli are influenced by 5 HT blockade series of experiments were done In one series the intestine was made tachyph

to 5 HT by i a infusions of this drug in increasing doses In the other series a 5 HT blocking drug dihydroergotamine (DHE) was administered in doses not causing α receptor blockade Due to its inavailibility bromo LSD was not used in this study (*cf* Biber *et al* 1971)

In both of the above mentioned experimental series it was clearly demonstrated that once an intestinal 5 HT blockade had been obtained intestinal vasodilatation could not be elicited either by i a infusion of hormones or by mechanical or electrical field stimulations A gradual return of the vasodilatory responses to all the stimuli was also observed after the period of blockade Thus one may conclude that all the procedures which induced intestinal vasodilatation in this study seem to act through vascular smooth muscle control mechanisms that are dependent on an intact 5 HT receptor function

Additional experiments in which nerve conductivity was blocked by tetrodotoxin were also performed to test whether the intestinal vasodilatations were mediated *via* nervous pathways In agreement with earlier studies from this laboratory the dilatations induced by mechanical and electrical field stimulations were blocked by tetrodotoxin (Biber *et al* 1971 1973 a) while the hormonal effects were unaffected by this drug Thus one may conclude that the hormonal vasodilatations are not nervously mediated while those elicited by mucosal mechanical stimulation and electrical field stimulation probably are

The latter conclusion poses the question of whether 5 HT acts as a transmitter substance in the suggested intramural nervous reflex arch (see Fig 6) 5 HT serves as transmitter substance in the brain (see *e g* Dahlstrom and Fuxe 1964 Carlsson 1967 Anden 1971) and it has been suggested to play a neurohormonal role in the gastrointestinal tract *e g* in the nervous control of intestinal motility (see *e g* Bulbring 1961 Bulbring and Gershon 1968 Gershon 1970) However to establish 5 HT as a transmitter in connection with intestinal vascular control several criteria have to be satisfied

1 The substance and the enzymes necessary for its formation and breakdown must be present in the nerve cell The presence of 5 HT in the gut wall has been clearly demonstrated where the mucosal enterochromaffin cells contain the greatest amounts (Erspamer 1966 a Vialli 1966) Some investigations also strongly suggest the presence of 5 HT in close association with the intramural intestinal plexa (Tafuri and Raick 1964 Gershon and Ross 1966 Hammarstrom *et al* 1966 Gershon and Altman 1971 Robinson and Gershon 1971) the amount however being small (Gershon 1970) Other authors have not been able to demonstrate the presence of 5 HT in intestinal nerve plexa (Norberg 1964 Read and Burnstock 1969) but have applied less sensitive techniques Enzymes necessary for the formation and breakdown of 5 HT are however present in the intestinal wall (Hagen and Cohen 1966) presumably also in intestinal nervous structures (Gershon and Ross 1966 Gershon 1970 Gershon and Altman 1971)

2 The substance proposed as a transmitter must be released from the axon when the nerve is activated Observations made in this study provide evidence for such a

release of 5 HT during the hyperemia elicited by transmural electrical field stimulation (Fig 5) while no release occurred during a vasodilatation caused by papaverine Earlier studies have shown that the plasma concentration of 5 HT increases upon mechanical mucosal stimulation (Burks and Long 1966) However none of these investigations provide evidence that the increased 5 HT output from the intestine actually emanates from intestinal nervous structures

3 The effect of the transmitter must be mimicked by the exogenous application of the substance As regards 5 HT and the intestinal vascular bed this criterion has also been experimentally tested in this laboratory (Biber *et al* 1973 b c) and similar effects of i.a. injected 5 HT and of electrical field stimulation were demonstrated on all vascular parameters studied However the vasodilator effect of injected 5 HT is blocked by tetrodotoxin suggesting that this vascular reaction is nervously mediated

4 Drugs which reduce or potentiate neurogenic responses should similarly affect the response to the exogenously applied substance As shown in this study the vasodilatations even upon transmural electrical stimulation and mechanical mucosal stimulation are blocked by 5 HT tachyphylaxis and by 5 HT receptor blockers in doses that abolish the effects of i a injected 5 HT No potentiating drugs have so far been tested

To summarize most criteria for 5 HT acting as a neurotransmitter in vascular control mechanisms in the gut wall seem to be fulfilled However the difficulty in differentiating between 5 HT released from intestinal nervous structures or from enterochromaffin cells makes a definitive conclusion impossible If 5 HT is a neuro transmitter the available experimental data seem to indicate that it is not released at the effector cell (*i e* at the vascular smooth muscle) but in a synapse (Fig 6) since exogenously applied 5 HT apparently acts *via* a nervous structure (Biber *et al* 1973 b) Additionally it should be pointed out that all the results summarized above implying 5 HT as a transmitter may be adequately explained also by the release of 5 HT from enterochromaffin cells and consequent stimulation of mucosal nervous receptors in an intramural reflex arch (see Fig 6)

Previously the participitation of CCK and secretin in the functional hyperemia of the gut was proposed and substantiated experimentally by Fara and coworkers (1972) They also provided evidence that this type of intestinal hyperemia was not mediated *via* cholinergic or adrenergic receptors nor *via* a direct effect of the hormones on the vascular smooth muscles In the present study a further attempt was made to elucidate the mode of vasodilator action of the gastrointestinal polypeptide hormones on the intestinal vascular bed It was shown that blocking the vascular effects of exogenously administered 5 HT also abolished the vascular effects of the hormones These effects were however evidently not nervously mediated (see above) and the hormonal effects seem therefore not to be mediated *via* the same mechanism as the transmural field stimulation This conclusion is at variance with the results recently reported as regards the hormonal control of intestinal motility Thus Vizi *et al* (1973) provided experimental support for the view that the stimu

lators effects of CCK and gastrin are nervously mediated the hormones causing a nervous release of acetylcholine

The true significance of 5 HT in the hormonally induced vasodilatations is unknown at present It should be recalled that 5 HT and polypeptide hormones are belived to be stored in the same dense intracellular granules in gastro-intestinal *enterochromaffin and enterchromaffin like cells (see e g Thompson 1971) Further*more intraduodenal instillation of acid causing an intestinal vasodilatation ascribed to the effects of released cholecystokinin and secretin (Fara *et al* 1972) is associated with decreased 5 HT stores in the intestinal tissue (Resnick and Gray 1962) Increased portal blood contents of 5 HT following feeding have also been reported in dogs (Smith 1958 Black *et al* 1959) To what extent and how these changes in the intestinal 5 HT content are related to the intestinal hormones has yet to be elucidated

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# Cardiovascular Responses to Acute Mental 'Stress' in Spontaneously Hypertensive Rats

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## Abstract

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Spontaneously hypertensive rats (SHR) aged 7 months (manifest hypertension) or 10—11 week (prehypertensive) and renal hypertensive rats (RHR nonhereditary hypertension) were compared with normotensive rats (NCR) concerning cardiovascular responses to mental stress Blood pressure and heart rate were followed in pairs of awake SHR—NCR and RHR NCR while defence reactions were provoked by alerting stimuli (light noise vibration) The tachycardia here involving both accentuated sympathetic and centrally suppressed vagal discharge reflected the intensity of neural activation and the pressure rise the imposed load on heart and vessels In both respects the SHR groups responded decidedly stronger than NCR and RHR also after either adrenergic or vagal cardiac blockade Since SHR and NCR hearts responded equally to graded vagal stimulations or to isoprenaline the enhanced tachycardia responses reflected a truly intensified defence reaction in SHR Further SHR responded more often to mild stimuli than NCR suggesting a lower threshold for defence reaction and more frequently with clearcut defence reactions than NCR which exhibited vagal bradycardia responses in 30 % versus 5 % in SHR This hyperreactivity and apparent prevalence of intermittent sympathetic tonic patterns in SHR during alertness in turn tending to trigger structural vascular adaptation and manifest hypertension is evidently genetically linked and not secondary to hypertension being observed particularly in prehypertensive SHR but not in RHR

For decades neurohormonal influences particularly in the form of an enhanced tonic sympathetic discharge have been proposed to decisively contribute to the increased resistance in essential (primary) hypertension (cf Pickering 1961) Increases in neurohormonal discharge although usually more or less intermittent in nature characterize also several centrally integrated response patterns elicited by arousal or emotional stress Of particular interest is here the hypothalamic centre conveying the response to most types of alerting or stressful stimuli the defence reaction (Eliasson et al 1951 Abrahams Hilton and Zbrozyna 1960) It implies

a central inhibition of vagal tone associated with an intensified sympatho adrenergic discharge to heart and vessels—except the muscle ones—and resulting in a pressure rise secondary to an increased cardiac output that particularly favours the skeletal muscles

In man early phases of essential hypertension often resemble a mild defence reaction (*e g* Brod *et al* 1962 Julius Pascual and London 1971) and hyperreactivity to emotionally disturbing stimuli has been reported (*e g* Kalis *et al* 1957 Nestel 1969 Lorimer *et al* 1971) — When the hypothalamic defence area in rats is exposed to often repeated topical stimuli the transient pressure rises can gradually lead to a more persistent pressure elevation (Folkow and Rubinstein 1966) also occurring in animals exposed to prolonged environmental stress (*e g* Hudak and Buckely 1961 Henry Meehan and Stephens 1967, Henry and Cassel 1969 Smookler and Buckley 1969 Herd *et al* 1969)

Despite such results neurogenic influences have not been generally accepted as an important element in the pathogenesis of essential hypertension mainly because resistance remains proportionally elevated also after regional sympathetic blockade compared with similarly treated controls Since most investigators appear to assume that this non neurogenic increase of resistance must reflect a persistently enhanced activity of the vascular smooth muscle there has been an intense search for other smooth muscle excitatory influences that might explain this assumed activity increase but largely in vain

However hemodynamic studies (*e g* Folkow 1956 Folkow *et al* 1973) suggest that the sustained resistance increase in primary hypertension is rather a matter of a rapidly established *structural* increase in wall/lumen ratio (structural autoregulation) by its very presence raising resistance over the entire range of smooth muscle activity compared with control even during maximal dilatation This alternative approach to the key problem of hypertensive disease in general again focusses the interest on central neuro-hormonal mechanisms in primary hypertension but now rather as generating transient pressor increases that serve to trigger adaptive changes in vascular design and thereby a sustained resistanse increase (*cf* Folkow *et al* 1970)

The experimental study of such problems has been greatly facilitated by the creation of spontaneously hypertensive rats (SHR Okamoto 1969) which provide research with the so far best animal model of essential hypertension This variant of primary hypertension like that in man (Pickering 1968) exhibits several hereditary elements predisposing for high blood pressure One of these elements may imply a raised centrally elicited neuro-hormonal activity in SHR and findings by Okamoto s group point in this direction (Okamoto 1969) Thus chronic stress leads to a larger pressure elevation in SHR than in similarly treated controls (Yamori *et al* 1969)

The present study was designed to explore whether SHR are inherently hyperreactive with particular respect to the defence reaction when initiated by sudden alerting stimuli Brief preliminary reports have been earlier presented (Folkow *et al* 1972 1973)

TABLE I *Panel A* Mean resting heart rate and blood pressure with SEM for older SHR, young prehypertensive SHR and RHR as compared with their respective NCR during basal conditions. The differences in heart rate and blood pressure between the hypertensive and normotensive animals in each group is expressed as a ratio with the level of significance indicated above.
*Panel B* Mean values with SEM of the individual reductions in heart rate and blood pressure in the respective groups of rats after infusion of the adrenergic $\beta$ receptor blocker alprenolol 10 mg/kg b w. The levels of significant difference from the preinfusion value as well as for the ratio hypertensive/normotensive are indicated above the respective figures.
*Panel C* Mean heart rate and blood pressure with SEM of the respective group after administration of both alprenolol 10 mg/kg b w and atropine 2 mg/kg b w reflecting the intrinsic heart rates of the various groups of animals. The difference between the groups is indicated as in panel A.
*Panel D* Mean values with SEM of the individual increases in heart rate and blood pressure from basal condition after administration of atropine 2 mg/kg b w to 14 *pairs of SHR—NCR* in age varying from 2.5—7 months. Levels of significance are labelled as in panel B.
*Levels of significance* ns = not significant x = $p < 0.05$ xx = $p < 0.005$ xxx = $p < 0.001$

| | OLDER SHR – NCR age 7 months | | | YOUNG SHR – NCR age 10 11 weeks | | | RHR – NCR age 12 13 weeks | | |
|---|---|---|---|---|---|---|---|---|---|
| BASAL CONDITIONS | SHR | NCR | SHR/NCR | SHR | NCR | SHR/NCR | RHR | NCR | RHR/NCR |
| A Heart rate beats/min | 380 8 | 398 10 | n.s. | 380 14 | 420 11 | $0.9^{x}$ | 425 12 | 415 9 | n.s. |
| Blood pressure mm Hg | 178 4 | 127 4 | $1.4^{xxx}$ | 137 3 | 117 3 | $1.2^{xxx}$ | 185 5 | 113 3 | $1.6^{xxx}$ |
| ADRENERGIC β RECEPTOR BLOCKADE | | | | | | | | | |
| B Δ Heart rate from basal, beats/min | $64^{xxx}$ 12 | $29^{xx}$ 9 | 2 2 | $65^{xxx}$ 10 | 20 8 | $3.2^{xxx}$ | 27 12 | 5 12 | s. |
| Δ Blood pressure from basal mm Hg | 8 6 | 2 3 | n.s. | 18 4 | 7 3 | $2.6^{x}$ | $14^{x}$ 5 | 5 3 | s |
| ADDITION OF ATROPINE | | | | | | | | | |
| C Intrinsic heart rate beats/min | 390 8 | 436 8 | $0.9^{xxx}$ | 434 10 | 486 11 | $0.9^{xxx}$ | 481 11 | 496 7 | |
| Blood pressure mm Hg | 171 7 | 131 4 | $1.3^{xxx}$ | 129 4 | 114 4 | 1.1 | 179 6 | 112 4 | $1.6^{xxx}$ |

| ATROPINE ALONE, 2 mg/kg | SHR | NCR | SHR / NCR |
|---|---|---|---|
| D Δ Heart rate from basal, beats/min | $57^{xxx}$ 8 | $79^{xxx}$ 7 | $0.7^{x}$ |
| Δ Blood pressure from basal mm Hg | 2 1 | 6 2 | n.s. |

## Results

*I Basal resting conditions*

Table I illustrates the resting levels of heart rate and blood pressure in the 3 groups of hypertensive rats (young SHR older SHR RHR) as compared with matched normotensive controls. The table gives A) The absolute values for SHR (RHR) and NCR and the ratio between them before any drugs were given B) The absolute changes induced by selective $\beta$ receptor blockade (alprenolol 10 mg kg) C) The intrinsic heart rate revealed when 2 mg/kg of atropine had blocked also the vagal heart influence Finally D) the absolute changes in heart rate and blood

pressure induced by selective vagal blockade in 14 pairs of SHR—NCR aged 11 weeks to 8 months

1) *Heart rate* Both SHR groups exhibited slightly lower resting heart rates than their normotensive controls during the prevailing resting conditions although this difference was statistically significant ($p < 0.05$) only for young SHR. RHR on the other hand tended to have slightly higher heart rates than their matched NCR but this difference was not significantly different (A Table I)

After $\beta$ receptor blockade (B Table I), the resting heart rate of both young and older SHR was reduced considerably more ($p < 0.001$ and $p < 0.05$ respectively) than that of their matched controls, the immediate reductions in steady state conditions calculated from the individual experiments being $65 \pm 10$ *vs* $20 \pm 8$ beats/min for young SHR and NCR respectively and $64 \pm 12$ *vs* $29 \pm 9$ beats/min for older SHR and NCR During the period of measurement subsequent to the $\beta$ receptor blockade the mean resting heart rate was for young SHR—NCR $335 \pm 13$ and $387 \pm 14$ beats/min, respectively and for older SHR—NCR $297 \pm 9$ and $355 \pm 8$ beats/min both differences between SHR—NCR being statistically significant ($p < 0.01$) RHR and their matched NCR on the other hand did not differ significantly after $\beta$ receptor blockade concerning either resting heart rate or its reduction as a result of the blockade

Since alprenolol exhibits a slight inherent $\beta$ receptor stimulating effect 8 pairs of older SHS—NCR were given propranolol (3 mg/kg) instead After this type of $\beta$ receptor blockade the resting heart rate values were $291 \pm 7$ for SHR and $331 \pm 3$ beats/min for NCR thus slightly lower values than after alprenolol but otherwise similarly related — The efficiency of the $\beta$ receptor blockade was tested by i a administration of 0 1 and 0 5 $\mu g$ isopropylnoradrenaline (INA) before and after Aptin No significant difference between SHR and NCR was observed concerning responsiveness to INA neither before nor after alprenolol Thus 0 1 $\mu g$ INA increased heart rate in SHR by $70 \pm 8$ beats/min and by $88 \pm 12$ beats/min in NCR before alprenolol while the corresponding increases after alprenolol were only $9 \pm 2$ and $8 \pm 5$ beats min respectively The corresponding figures for 0 5 $\mu g$ INA were $131 \pm 13$ and $129 \pm 12$ beats/min before Aptin and $19 \pm 3$ and $18 \pm 3$ beats/min after Aptin

The tonic vagal inhibitory influence on heart rate was blocked by atropine (2 mg/kg in addition to the earlier blockade of cardiac sympathoadrenal influences thus reflecting in relative terms the intrinsic heart rate (C Table I) The figures were for older SHR and NCR, $380 \pm 8$ and $436 \pm 8$ beats/min respectively for young SHR and NCR $434 \pm 10$ and $486 \pm 11$ and for RHR and NCR $481 \pm 11$ and $496 \pm 7$ respectively Thus both young and older SHR exhibited some 10 % lower intrinsic heart rates than their matched normotensive controls ($p < 0.001$) while RHR—NCR did not differ significantly in this respect

The extent of the tonic vagal heart influence in SHR—NCR during rest is perhaps best reflected in the group of young and older SHR—NCR receiving only atropine (2 mg/kg The resting heart rate before atropine was here $371 \pm 10$ for

SHR and 411 ± 10 beats/min for NCR their blood pressures being 165 ± 7 and 115 ± 2 mm Hg After atropine SHR exhibited a significantly less pronounced cardiac acceleration than NCR ($p < 0\,05$) the mean of all immediate increase being 57 ± 8 and 79 ± 7 beats/min respectively It resulted in resting heart rates after selective vagal blockade of 425 ± 8 beats/min in SHR *vs* 487 ± 7 in NCR a significant difference ($p < 0\,05$)

Thus to judge from these pharmacological interferences with the vagal and sympathetic control of the heart SHR displayed a somewhat more pronounced sympathetic influence than NCR during the prevailing resting conditions while the vagal tonic influence was somewhat less pronounced In return the intrinsic heart rate was considerably lower in SHR than in NCR In all these respects RHR and NCR did not differ significantly

Concerning variability in heart rate reflecting the degree of lability in cardiac neurogenic control during these standardized resting conditions older SHR and RHR did not differ significantly from their paired NCR However the young SHR showed evidence of a more labile neurogenic cardiac control during rest than their matched controls to judge from the significant difference of 2 6 ± 0 6 ($p < 0\,001$) in the standard deviations of their respective means (see Methods) This difference was greatly reduced by $\beta$ receptor blockade and abolished when also atropine was given procedures which also in the other animal groups reduced respectively abolished the more modest variability in heart rate during resting conditions

2 *Blood pressure* Measured intraarterially during the prevailing resting conditions arterial blood pressure was 178 ± 4 mm Hg in older SHR and 127 ± 4 mm Hg in their matched normotensive controls a difference of some 50 mm Hg The young SHR, being in the early prehypertensive phase (10—11 weeks old) had a resting blood pressure of 137 ± 3 mm Hg *vs* 117 ± 3 mm Hg for their matched controls a difference of only some 20 mm Hg The 12—13 weeks old RHR exhibited the highest mean blood pressure of all the groups 185 mm Hg

The changes in resting blood pressure as a result of alprenolol and/or atropine treatment are shown in Table I comparing sections A B C and D In the older SHR—NCR $\beta$ receptor blockade did not significantly change the resting blood pressure while it caused a significant drop (18 ± 4 mm Hg) in young SHR and a smaller one in their matched normotensive controls (7 ± 3 mm Hg) the pressure drop in SHR being significantly greater than that in NCR A small but significant pressure drop was seen also in the young RHR though it did not differ significantly from that of their matched controls Atropine whether alone or in combination with $\beta$ receptor blockade did not further change the resting blood pressures appreciably in any of the groups (C and D Table I)

*II Effects of alerting stimuli*

The cardiovascular responses to the three types of alerting stimuli used were taken as a measure of the intensity of the centrally induced defence reaction Judged on such a basis vibration proved to be the most potent stimulus and flashing light tl

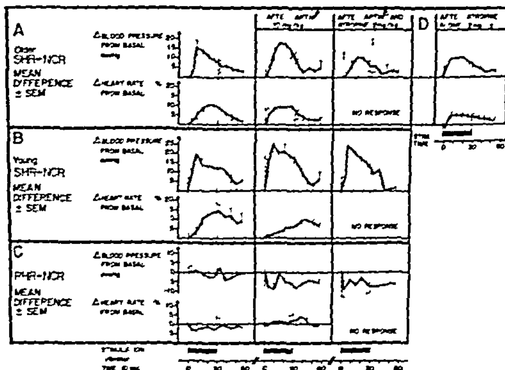



Fig 1 The mean difference between elder SHR and NCR (A) between young prehypertensive SHR and NCR (B) and between RHR and NCR (C) are shown concerning heart rate and blood pressure responses during 30 s exposure to vibrations and the 30 s poststimulatory period From the left is shown the responses before drug administration then after administration of a β adrenergic antagonist alprenolol Aptin® 10 mg/kg) and after both alprenolol and atropine (mg/kg b w) administration To the far right (D) is shown the situation when 14 pairs of SHR NCR aged 2.5 months are given atropine (2 mg/kg) alone

Differences in blood pressure response for each group of animals (A B C D) is based on the mean of the differences in increase from baseline in each pair of hypertensive and normotensive rat each third second The vertical bars indicate SEM of the mean difference Differences in heart rate are first expressed as the difference in percentual change from baseline for each pair of hypertensive and normotensive animals Then the mean of all these differences are presented as curves with SEM indicated as vertical bars

weaker one As earlier outlined the defence reaction is among other things characterized by a pressure rise associated with a cardiac acceleration. This heart rate increase involving both accentuated sympathetic and inhibited vagal tone will closely reflect the magnitude of the neurogenic shifts while the pressure rises of course in a direct way reflect the load imposed on heart and vessels

Fig 1 and Table II summarize in 2 different ways the results of exposing the animals to alerting stimuli Thus Fig 1 presents the compiled *difference in response curves* concerning heart rate and blood pressure changes to vibration between the three groups of hypertensive rats and their matched controls first before drug administration then after β receptor blockade and finally after β-receptor blockade in combination with vagal blockade The far right part of Fig 1 illustrates the same

| | OLDER SHR NCR age 7 months | | | | | | YOUNG SHR NCR age 10 11 week | | | | | | RHR NCR age 12 13 week | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TYPE OF ALERTING STIMULUS | Δ Blood pressure from basal peak response, mmHg | | | R change from basal peak response % | | | Δ Blood pressure from basal peak response, mm Hg | | | R change from basal peak response % | | | Δ Blood pressure from basal peak response, mm Hg | | | change from basal peak response % | | |
| | SHR | NCR | [illegible] | SHR | NCR | [illegible] | SHR | NCR | [illegible] | SHR | NCR | [illegible] | RHR | NCR | [illegible] | RHR | NC | [illegible] |
| FLASHING LIGHT | 7 4 | 2±1 | n.s. | 5±2 | 3±2 | | 12 2 | 3 1 | | 4 2 | 2 1 | x | 3 2 | 5 2 | | 1±1 | 2 1 | x |
| LOUD NOISE | 17 2 | 6 2 | | 6±2 | 1±2 | | 24 2 | 3±1 | xxx | 7 2 | 2 2 | | 5 2 | 8 3 | n.s. | -4 2 | -5 2 | n.s. |
| VIBRATIONS | 20 3 | 5 2 | | 11 2 | 2 2 | | 25 | 6 2 | xxx | 11 3 | -4 2 | xx | 6 4 | 10 2 | n.s. | 3 2 | 4 2 | n.s. |
| | AFTER ADMINISTRATION OF ADRENERGIC β RECEPTOR BLOCKING AGENT APTIN® 10 mg/kg | | | | | | | | | | | | | | | | | |
| FLASHING LIGHT | 7 3 | 2 2 | n.s. | 4 1 | 2 1 | n.s. | 14 3 | 8 6 | n. | 4 3 | 2 1 | x | 8 2 | 8 2 | n.s. | 5 3 | 3 1 | n.s. |
| LOUD NOISE | 19 2 | 6 1 | xxx | 9 2 | 4 3 | n.s. | 22 3 | 8 2 | xx | 5 1 | 4 1 | | 13 2 | 13 2 | n.s | 4 2 | 3 1 | n.s. |
| VIBRATIONS | 24 4 | 11 3 | | 10 3 | 1 2 | xx | 25 3 | 8 2 | xxx | 7 1 | 2 2 | | 10 2 | 16 3 | n.s. | 3 2 | 2 2 | n.s |
| | AFTER APTIN® 10 mg/kg AND ATROPINE 2 mg/kg | | | | | | | | | | | | | | | | | |
| FLASHING LIGHT | 4 1 | 2 2 | x | o esponse | | | 12 1 | 6 2 | | no re ponse | | | 10 3 | 9 2 | n.s. | no response | | |
| LOUD NOISE | 14 3 | 8 3 | | no response | | | 3 2 | 9 1 | xxx | no esponse | | | 5 2 | 12 1 | n.s. | no r sponse | | |
| VIBRATION | 19 2 | 11 2 | | no espon | | | 26 3 | 9 | xx | no esponse | | | 10 3 | 17 2 | n.s. | no esponse | | |

TABLE II Presents the heart rate and blood pressure changes in the three groups of hyper tensive rats and their NCR when exposed to alerting stimuli first before and then after administration of alprenolol (Aptin®) 10 mg/kg b w and finally after both alprenolol and atropine 2 mg/kg b w The largest deviation (peak value) in blood pressure during the 30 s stimulation period expressed in mm Hg from prestimulatory level is presented for each group of animals with SEM and levels of significance Similarly the peak heart responses expressed as percentage change from prestimulatory level are given The difference between hypertensive and normotensive animals (sign SHR—NCR) is based on the level of significant difference not only of the peak response but of more than 6 of the 10 time matched observations during the total stimulation period
*Level of significance* ns = not significant x = $p < 0.05$ xx = $p < 0.005$ xxx = $p < 0.001$

difference between the groups of SHR—NCR both young and older that were given atropine only — It is clear from all these curves that both blood pressure and heart rate responses to vibration were throughout more pronounced in the two SHR groups compared with their controls while RHR did not differ significantly from their controls

*Table II* summarizes instead the mean *peak responses in blood pressure and heart* rate during the 30 s period of stimulation with all three kinds of alerting stimuli in the three groups of paired animals first before and then after drug administration It shows again that SHR particularly the young ones responded more vividly con cerning increases of both heart rate and pressure as compared with their matched controls while RHR did not differ significantly from their controls With respect to the heart rate changes the compiled figures may give the impression that the genetically normotensive rats *i e* all NCR and RHR reacted only little to the alerting stimuli used and sometimes the peak response was negative Control of the individual experiments revealed that 37 % of NCR responded with bradycardia to the most potent stimulus vibration while 57 % showed cle

tachycardia and the remaining 11 % no substantial heart rate change RHR showed very similar figures 38 % 56 % and 6 % respectively In contrast older SHR exhibited tachycardia in 94 % and bradycardia in only 6 % the corresponding figures for young SHR being 85 % and 5 %, with 10 % showing no significant heart rate changes It is obvious that such diverging responses the significance of which will be discussed later tend to cancel out in the compiled mean values and in NCR and RHR result in considerable lower mean values than in SHR concerning increases in heart rate However this by no means explains the entire difference between SHR and NCR since a comparison of only those SHR and NCR which throughout exhibited clearcut tachycardia responses revealed significantly more pronounced such responses in SHR Further the animals given only atropine revealed that the mentioned bradycardia was essentially of vagal origin since all animals exhibited tachycardia responses after atropine but again SHR significantly more so than NCR

Also after selective *β* receptor blockade almost all SHR and most RHR and NCR responded to the alerting stimuli with cardiac acceleration then obviously mediated by the vagal nerves as is typical for the defence reaction with its central suppression of vagal cardiac tone Also these tachycardia responses were significantly larger in SHR (see Table II Fig 1) demonstrating a more pronounced central inhibition of vagal tone than NCR along with the defence reaction while RHR and NCR did not differ significantly in this respect

To check that this difference in heart rate response between SHR and NCR was of central nature and not merely a matter of *e g* a peripheral neuroeffector difference the cardiac responses to graded right vagal stimulations were compared in SHR—NCR after *β* receptor blockade and vagal decentralization At physiological frequencies between 0 up to 10 Hz SHR and NCR responded identically but SHR reached the maximal level of bradycardia at a slightly lower rate of stimulation than NCR (12 *vs* 14 Hz probably because of a somewhat lower initial heart rate The maximal degree of cardiac deceleration was the same for SHR and NCR (107 ± 12 and 102 ± 12 beats/min respectively) and SHR—NCR therefore exhibited virtually identical characteristics concerning the vagal influence on heart rate It follows that the more pronounced tachycardia of SHR in response to alerting stimuli after *β* receptor blockade reflects a more pronounced central vagal inhibition Similarly while SHR displayed more vivid heart rate (and pressure) responses than NCR to alerting stimuli after atropine alone when the cardiac changes were entirely mediated by adrenergic mechanisms (Fig 1 Table II) their largely equal responses to INA indicate that this difference is not a matter of any difference concerning *e g* adrenergic receptors or cardiac effectors

After both atropine and *β* receptor blockade all heart rate responses to alerting stimuli were abolished in all groups indicating a complete elimination of extrinsic cardiac influences However Fig 1 shows that the pressor responses were still considerably larger in both SHR groups as compared with their controls while RHR and NCR did not differ significantly

Transient pressor responses to alerting stimuli are of interest not only with respect

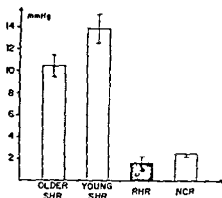



Fig 2 Illustrates the mean blood pressure elevation during 30 s exposure to loud noise and the poststimulatory 30 s period in older SHR young SHR and in RHR (18 animals in each group) as compared with all NCR used in the study (72 animals)

to their peak effects as illustrated in Table II and partly in Fig 1 but also concerning their duration and of course frequency of occurrence Clearly all these aspects contribute to the increase of the average pressure load imposed on the cardiovascular system by environmental stress thus together determining the impact of neuro-hormonal mechanisms The frequency of stress-induced cardiovascular reactions occurring in the normal environment of the animals can for obvious reasons not be evaluated from the present experiments but they illustrate some aspects of the *duration* of such responses beside their *intensity* and might also give some hints concerning their *frequency* in SHR compared with NCR Fig 1 indicates that the exaggerated blood pressure and heart rate increases in SHR tend to subside more slowly than in NCR during the 30 s poststimulatory period particularly in young SHR In addition Fig 2 gives the average blood pressure increase during the 30 s stimulation and 30 s poststimulation periods for older and young SHR for RHR and NCR utilizing noise as alerting stimulus Particularly young SHR respond with an average pressure rise that is some five times as high as in NCR during the mentioned period of time while RHR do not differ significantly from NCR in this respect

The possible presence of a difference between SHR and NCR concerning also the frequency of neurogenic cardiovascular responses to environmental stimuli in general is illustrated by the heart rate responses to the weakest alerting stimulus used light flashes If heart rate changes above 5 % in the individual animals are arbitrarily considered as a sign of a barely suprathreshold response to light SHR responded 35 % more frequently than NCR and of these positive responses tachycardia was seen 65 % more frequently in SHR RHR if anything responded l frequently than their controls in this respect These results suggest hat SHR respond with significant cardiovascular responses in their natural environment frequently than NCR

## Discussion

Though the pathogenesis of essential hypertension is indeed a matter of intense dispute authorities do agree on a few points among them that the raised resistance is ultimately dependent on hereditary elements though these are still of unknown nature (see Pickering 1968) As they for obvious reasons are very difficult to identify in man the development of rat strains with spontaneous genetically linked hypertension with almost 100 % incidence such as SHR (see Okamoto 1969) and the New Zealand variety (Smirk and Hall 1958) has meant a great step forward for studies of primary hypertension in general Genetic studies here suggest the involvement of relatively few independent but major genetic elements acting additively (*e g* Louis *et al* 1969 Tanase *et al* 1970)

Extensive studies have been carried out concerning neuro-hormonal mechanisms in SHR pointing to an intensified activity not only of the sympathoadrenal system but also of *e g* the ACTH-corticoid and TSH thyroid systems (Okamoto 1969 Tabei *et al* 1972) as occurs also in normal organisms in situations of *e g* mental stress In such situations the excitatory defence reaction with its cortical arousal and specific hormonal and autonomic somatomotor discharge pattern preparing for fight or flight (see Introduction) usually dominates behaviour Early phases of essential hypertension often exhibit a neurogenic circulatory change strongly suggesting the presence of a mild defence reaction blood pressure being raised in combination with increases of heart rate and cardiac output favouring skeletal muscle

Towards such a general background the present study was designed to test whether SHR both young prehypertensive and older with established hypertension display inherently exaggerated defence reactions to graded alerting stimuli as compared with matched NCR and rats with non genetic (renal) hypertension (RHR) It was also explored whether SHR display milder signs of such a pattern even during rest —though then certainly a relative one because of exposure to an artificial laboratory environment even if the animals were otherwise left undisturbed Further if this is the case it is of particular interest to establish beyond doubt that it really can be ascribed to an enhanced neurogenic drive which is most easily traced in intact organisms concerning the impact on heart rate because of its close relationship to the frequency of autonomic fibre discharge (*cf* Folkow 1960 a)

Even during anesthesia young SHR display a hyperkinetic hemodynamic pattern (Pfeffer and Frohlich 1973) similar to that in early essential hypertension in man Although operative procedures thoracotomy with artificial respiration and carotid occlusion have to be considered the results of Pfeffer and Frohlich indicate that the tonic parasympathetic discharge is reduced and the sympathetic discharge enhanced in SHR compared with NCR Also awake SHR left largely intact in a semidark silent though no doubt foreign) environment display a reduced vagal tone and an accentuated sympathetic discharge to the heart according to the present results just as in resting awake subjects with early labile hypertension (Julius Pascual and London 1971

Particularly concerning the cardiovascular responses to alerting stimuli the heart rate changes are suitable for judging the type and extent of the shifts in neural discharge However for judging the consequent load imposed on heart and vessels the pressure rises are of course the relevant indicator The present results demonstrate that the neurogenic discharge to alerting stimuli and the resulting increases of pressure are both considerably augmented in SHR compared with NCR certainly concerning extent and probably concerning duration as well This augmentation obviously precedes hypertension in SHR and is not a consequence of a hypertensive state being particularly marked in young nearly normotensive SHR but absent in renal hypertension Furthermore SHR also appear to display a somewhat reduced threshold concerning responses to such stimuli as judged by the enhanced lability in resting heart rate in young SHR and their more frequent cardiac responses to flashing light Thus al o *the frequency of naturally occurring pressure rises may be* higher in SHR than in NCR since any species is no doubt frequently exposed to natural environmental stimuli tending to elicit arousal

The augmented changes in heart rate in SHR compared with NCR involved both a more pronounced central inhibition of vagal tone and an intensified central excitation *of the adrenergic heart supply These augmented heart rate responses in* SHR cannot be ascribed to differences in efferent cardiac innervation in cardiac effector cells or their cholinergic and adrenergic receptors because SHR and NCR exhibited virtually identical heart rate responses to graded vagal stimulations and to isopropylnoradrenaline administration

*In* addition an element of centrally elicited vagal bradycardia was more often intermingled with the excitatory adrenergic reactions in NCR and RHR than in SHR though seen in all groups as described in Results It is known that the organism may choose between several autonomic response patterns in situations of mental stress where some exhibit a vagotonic component (see Folkow and Neil 1971) and the element of vagal bradycardia may reflect the involvement of such other patterns perhaps competing with the more throughout excitatory defence reaction If so NCR—RHR are obviously more prone to respond in such a vagotonic direction than SHR The old debate of possible differences between human individuals with respect to sympathicotonic and vagotonic responses during mental stress should here be recalled

The described hyperreactivity of and the prevalence for the hypothalamic defence reaction in SHR is however only one expression of a more general shift in emotional balance and behaviour in SHR compared with NCR Thus their tendency of biting off the tail catheter and biting the investigator too for that matter was far more frequent *Several earlier investigations utilizing a variety of* behaviour tests point in the same general direction (*e g* Shimamoto and Nagaoka 1972 Takaori *et al* 1972)

As outlined in Introduction often repeated defence reactions can gradually lead to more persistent pressure elevations also in inherently normotensive animals *a* in *mice at least* even *induce* several *of* the degenerative lesional stigmata,

later stages of severe hypertension in man (Henry and Cassel 1969) The hormonal links forming an integral part of the defence reaction such as enhanced catecholamine and corticoid secretions neurogenic involvement of the renin angiotensin aldosteron system *etc* are here likely to be of great importance beside the more direct pressure load imposed by the neurogenic influence on heart and vessels Nevertheless such extrinsic neurohormonal changes alone provoked by environmental stimuli are probably not enough in most cases to fully match the situation in SHR or essential hypertension of man where several genetic predisposing elements contribute It is therefore not surprising that the hypertensive state is usually more modest and perhaps less persistent when neuro hormonal discharges are frequently imposed on inherently normotensive organisms by environmental stress Additional elements are obviously needed as suggested by the polygenetic inheritance of hypertension in both SHR and man one being that resulting in a raised neurohormonal drive as discussed above

Another element which might be genetically influenced in SHR is the adaptive structural change of the resistance vessels shown to occur not only in SHR and essential hypertension of man but in renal hypertensive rats (RHR) as well (see Folkow *et al* 1973) This structural autoregulation increases the wall/lumen ratio of the resistance vessels mainly by media hypertrophy (*cf* Suwa and Takahashi 1971) and it occurs within few weeks at least in rats enhancing vascular resistance for any given smooth muscle activity compared with control even during maximal dilatation Once established this structural autoregulation appears to be pronounced enough to almost alone maintain the raised resistance during rest without necessitating any *sustained* rise in smooth muscle activity at least not in primary hypertension

Some results might indicate that this general tendency of heart and vessels to adapt structurally to increases of the pressure load is slightly more pronounced in SHR than in NCR—RHR (*cf* Folkow *et al* 1973) though this is for several reasons very difficult to prove beyond doubt

In summary then the present results reveal a central hyperreactivity in SHR compared with NCR (and RHR) SHR displaying more intense and perhaps also more prolonged defence reactions to brief standardized alerting stimuli They might in addition exhibit more frequent such responses in their natural environment to judge from the higher frequency of clearcut cardiac responses to the weakest stimulus used (light flashes) Moreover SHR appear to be more prone than NCR—RHR to induce clearcut defence reactions instead of patterns involving vagotonic components in moments of mental stress This hyperreactivity and apparent prevalence concerning a central neuro hormonal pattern which because of its considerable pressure rise is particulary apt to be a potential trigger mechanism for hypertension evidently reflects a genetically linked difference to NCR Further the fairly rapid structural adaptation of heart and resistance vessels to increases in load will lead to an established hypertensive state once the neurohormonal trigger mechanisms have started the ball rolling (Folkow 1960 b) Such a chain of events

here simplified but in reality certainly most complex and individually varying implies a multifactorial combination with a tendency of vicious circle influence not unrelated to the ideas inherent in Page s mosaic theory (1949)

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Acta physiol scand 1974 90 699—702

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# Diurnal Variation of Serum Immunoassayable Thyrotropin (TSH) Concentration in the Rat

By

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## Abstract

Leppalloto J T Ranta and J Tuomisto *Diurnal variation of serum immuno assayable thyrotropin (TSH) concentration in the rat* Acta physiol scand 1974 *90* 699—702

The diurnal variation in the immunoassayable TSH content of sera of female and male adult rats under conditions of 12 hours light and 12 h darkness was determined In the females the TSH level rose gradually from 11 p m to 3 p m and then declined in the males the level rose from 7 p m to 11 a.m and then declined Scattering of individual values was great (mean coefficient of variation was 0 45—0 46) For the estimation of the serum TSH level these determinants should be known

Daily occurring changes of the environment are known to modify neural and endocrine functions It has been shown that the motor activity of the rat is highest during the night whereas the plasma corticosterone level is high in the afternoon when motor activity is low (Guillemin *et al* 1959 Ader and Friedman 1967) The details of the diurnal fluctuation of the function of the pituitary thyroid axis in this species are somewhat controversial The plasma TSH concentration has been claimed to be maximal either early in the morning (Jobin and Samel 1964 Ducommun *et al* 1966) or in the afternoon (Singh *et al* 1967) The present report deals with *the diurnal variation of serum TSH in the rat as shown with a radioimmuno* assay method

## Materials and methods

Sprague Dawley rats 56 female and 36 male animals weighing 180—220 and 220—300 g respectively were fed *ad lib* on a pelleted diet (iodine concentration about 0 6 mg/kg) and tap water For 10 days before the experiments the animals were kept in individual cages in a silent room at 25 C with artificial illumination from 7 a m. to 7 p m They were not handled at all during that time The cages were so arranged that a cage could be taken out of the room without disturbing the other rats

The animals were taken from the silent room and decapitated within 20 s Blood was collected in plastic test tubes No difference in blood TSH concentration was found betw

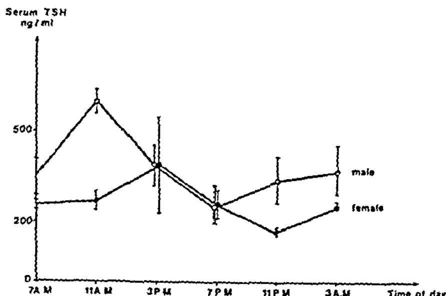



Fig 1 Diurnal variation of the serum TSH concentration in the rat Mean ± SE are given Number of animals females 8 at each point males 9 at 7 a m 6 at 3 7 and 11 p m. 5 at 3 a m and 4 at 11 a m Light on at 7 a m. light off at 7 p m.

serum and plasma or between arterial and venous blood The blood samples were taken at 4 h intervals beginning either at 11 a.m or at 7 p m Four animals were decapitated on each occasion The decapitation sequence did not affect the serum TSH concentration

8 female rats were anesthetized with pentobarbital (30 mg/kg) ip The first blood sample was drawn from the femoral vein and then 50 µg TRH (Ferring Ltd ) was injected i v into 4 animals and the rest received saline 30 min later the second blood sample was taken

All the serum samples were separated by centrifugation and stored at −20 C before estimation Serum TSH was measured individually from duplicate 100—200 ul samples by a double antibody radioimmunoassay A rat TSH kit was received as a gift from the NIAMD Rat Pituitary Program The results are expressed in ng/ml of a NIAMD Rat TSH RP 1 standard

Vaginal smears were taken from female animals and stained and studied under a light microscope Pro-oestrus oestrus *metoestrus* and dioestrus were separated according to Zarrow (Zarrow *et al* 1964) Student's test was used for comparisons

## Results and discussion

The serum TSH concentration was 386 ± 80 ng/ml (mean ± S E ) in rats anesthetized with pentobarbital before TRH injection and rose to 2230 ± 345 ($P < 0.01$) within 30 min after the injection In saline treated anesthetized animals the serum TSH concentration remained unchanged So its is evident that the radioimmunossay used measures TSH in serum

Diurnal fluctuation of the serum TSH concentration is shown in Fig 1 In the female rat the highest value 387 ± 158 ng/ml was seen at 3 p m and the lowest 167 ± 11 at 11 p m The latter differed significantly from values at 7 11 and 3 a m ($p < 0.01$) In the male rat the highest value was found at 11 a m. and the lowest

at 7 pm 596±40 and 258±63 The difference w  
0 01) The value at 11 a m also differed significantl  
and 11 p m ($p < 0.05$)

In this experimental schedule, light on from 7 a m  
TSH levels were seen between 11 a m and 3 p m. a  
and 11 p m This differs slightly from the earlier re  
1964 Ducommun *et al* 1966) who found the high  
level at 7 or 8 a m and the lowest at 7 p m A mo  
cult however because in those studies either the sa  
exact information was not available Singh *et al* (1  
glutination inhibition assay technique for TSH that  
TSH concentration was highest at 3 p m Their resul  
ours They did not state the precise duration of blood  
ether anesthesia which has recently been shown to  
(Martin and Reichlin 1972)

In the male rats the serum TSH concentration w  
animals The mean coefficients of variation were lar  
and 0 46 in the males) The variability found here wa  
agrees with a previous study in which the variation  
level between individual rats was likewise found  
Melander 1965)

In the female rats the relation of the serum TSH  
cycle was examined after exclusion of diurnal variati  
In this respect TSH differs from LH which has a pr  
1969)

Similarly it is unlikely that the serum TSH conc  
decapitation procedures because the procedure was  
disturbing the other animal It is emphasized that th  
cause any systematic change in the serum TSH conce

At the moment the cause of the lability of the  
known but may be associated with the existence of  
systems controlling the secretion of TSH (Tuomist  
seems to be inherent and must be taken into acco  
samples are pooled

In the present paper two significant determinants  
in the rat are documented firstly the large variati  
secondly the circadian fluctuation of the serum TSH  
blood concentration of this hormone these determ

We are grateful to the Rat Pituitary Hormone Dist  
providing the rat TSH RIA kit

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Acta physiol scand 1974 90 703—715

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# Longitudinal Propagation of Myogenic Activity in Rabbit Arteries and in the Rat Portal Vein

By

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## Abstract

BEVAN J A and B LJUNG *Longitudinal propagation of myogenic activity in rabbit arteries and in the rat portal vein* Acta physiol scand 1974 90 703—715

Longitudinal spread of induced activity was analysed in the rat portal vein and rabbit aorta and compared to propagation in the brachial radial ear mesenteric femoral and posterior tibial arteries of the rabbit Isometric force in spiral strips or intact preparations was measured during superfusion with Krebs solution The lower 20 per cent of the preparation could be selectively exposed to exogenous noradrenaline (NA) or to transmural field stimulation Propagation was inferred to occur when the amplitude of the local response relative to that when the entire preparation was stimulated exceeded the relative length of the locally stimulated tissue No propagation was found in the rabbit aortic strip Propagation was less in proximal than in distal muscular arteries The calculated distance of propagation was greatest in the rat portal vein The intact limb vessel preparations reponded to NA with phasic contractions In the ear artery here was propagation only of the first phase of this biphasic response These results show that different sections of the rabbit vascular tree display individuality in their ability to support propagation In general propagation in the arterial tree appears to be greatest in the smaller vessels

Studies of the morphological and physiological properties of blood vessels have convincingly demonstrated that the vascular smooth muscle in different parts of the cardiovascular system exhibit great variation in design and function One important variant found in isolated vascular preparations is the role of myogenic propagation of activity in the control and integration of vascular muscle contraction Cell to-cell propagation is pronounced in the portal mesenteric veins (*e g* Johansson and Ljung 1967 Ljung and Stage 1970) but appears to be only of small significance in most large artries (*e g* Burnstock and Prosser 1960 Bevan *et al* 1970)

In the present study a method of examining quantitatively longitudinal propagation of induced activity along isolated blood vessels is applied to the rabbit thoracic aorta the rat portal vein and several muscular arteries from the rabbit The analysis is based on such spread of activation as is reflected in a mechanical response There fore propagation in the various muscular arteries can be compared to one another

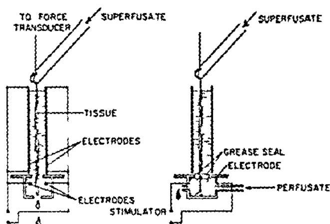



Fig 1 Schematic illustration of experimental arrangement Left panel Superfusion and electrode placement allowing NA exposure or transmural field stimulation of entire preparation Right panel Modification for selective NA application or transmural stimulation of lower part of preparation Superfusate was removed at the grease seal by suction Electrodes arranged horizontally for transverse current field NA was added to the perfusate Simultaneous addition of Evans blue allowed determination of the length of locally stimulated portion/total length of preparation at end of experiment

and to that in the rat portal vein and the rabbit aorta Since the comparison is between this functional consequence of propagation individualities in their respective electrophysiological properties can be disregarded

Some of the results were presented at the Fifth International Congress on Pharmacology San Francisco 1972 (Ljung and Bevan 1972)

## Methods

Isolated vascular preparations from white rabbits (appr 2 5 kg) of either sex and portal vein preparations from male rats (300 g) of the Sprague Dawley strain were studied The animals were stunned and bled A blood vessel was then isolated and excess tissue was removed under a dissection microscope

*Propagation experiment* A segment of the intact blood vessel or a spirally cut strip was attached with its distal end to the lower chamber of a lucite tissue holder schematically illustrated in Fig 1 The preparation was then pulled through a slit in the top of the chamber and the upper (proximal) end of the vessel was connected to a force transducer (Statham G 10B or Grass Ft 03) for isometric recording of contractile force on a Sargent recorder (SGR) fitted with a disc integrator or on a Grass polygraph For each group of vascular prepartions the passive tension which would give optimal responses was determined (range 150—1500 dyn) and applied in subsequent experimentation Since the contractile responses of an intact vessel resulted in a decrease in longitudinal force the polarity of the amplifier was inverted in such experiments (see Fig 4 5)

The suspended preparations were approximately 20 mm long The upper 80 per cent of the vessel was located between vertical platinum electrodes The lower 20 per cent was housed in a chamber which was fitted with horizontal electrodes for transmural nerve stimulation (see Fig 1) The left panel illustrates the experimental arrangement when responses of the entire preparation were studied The tissue was superfused with Krebs solution (Su 1968) at a local temperature of 37 C One minute responses to exogenous l noradrenaline (NA) were obtained by adding the drug to the superfusate Cocaine ($10^{-6}$ M) was added to the Krebs solution 30 min before and during exposures of the intact blood vessels to NA Nerve activation was achieved by transmural stimulation for 1 min between the pairs of electrodes on either side of the preparation Square wave impulses of 12 V amplitude between the electrode and of 0 5 ms duration from a Grass S4 stimulator were employed The impulses were monitored on an oscilloscope

The method for electrical stimulation of the lower portion of the tissue is illustrated in the right panel of Fig 1 The lower chamber was sealed off from the superfusate with a small

amount of silicone grease It was perfused with Krebs solution Exogenous NA could be added to the perfusate Activation of the local intramural nerve plexus was obtained with electrical field stimulation (0 5 ms 12 V) between the horizontal lower electrodes. In experiments with the rabbit aorta and the rat portal vein each stimulus was applied once In the experiments on other rabbit vessels each stimulus was applied twice The mean of these two latter responses was used in the calculations

In order to determine the portion of the preparation that was exposed to local stimulation Evans blue was added in a low concentration to the perfusate at the end of an experiment Subsequently the stained and the total length of the suspended tissue was measured with a pair of calipers

The response to local stimulation was expressed as a percentage of that response when the entire preparation was stimulated Propagation was concluded to occur when the percentage of the local/general contractile response (the relative amplitude) significantly exceeded the percentage of the stained/total length of the preparation when these values were tested for pair differences by Student s t test ($p < 0.05$)

*Length tension experiments* Preparations of the rat portal vein and spirally cut strips of the rabbit aorta were mounted at a passiv tension of 400 and 1500 dyn respectively in Krebs sol ion in an organ bath Isometric force was recorded with a Grass FT 03C force transducer (compliance ? × 10 $^{6}$ cm/dyn) on a Grass pol graph After a 1 h accomodation period during which the passive tension was adjusted p ak force responses to three 1 min exposures to NA 10 $^{7}$ 10 $^{6}$ and 10 M respectively w e recorded The lengths of the suspended preparations were then measured with a pair of alipers Subsequently the preparations were placed in Ca free Krebs solution for 30 min nd then their lengths reduced until zero tension was recorded The length tension relationshi for these inactive muscles were determind by stretching them by 0 25 or 0 5 mm length inc ments every 60 s The passive force at a given length was measured at the end of the stress relaxation period immediately prior to the next stretch

*Drugs* The following drugs were usel l noradrenaline bitartrate (Levophed Winthorp Laboratories) cocaine hydrochloride and Evans Blue (Harvey Laboratories)

## Results

*Rabbit thoracic aorta and rat portal vein — evaluation of the method*

In a series of experiments on rabbit thoracic aortic strips and rat portal vein preparations the responses to transmural nerve stimulation at different impulse rates were obtained using the electrodes in the lower chamber ( local nerve stimulation) The responses to local nerve stimulation were then expressed as a percentage of the responses to general transmural stimulation (field stimulation between the 2 sets of electrodes on either side of the total length of the tissue) Responses to graded concentrations of exogenous NA added to the perfusion fluid of the lower chamber ( local NA administration) were also measured and compared to the responses when the entire tissue was exposed to the same NA concentration ( general NA administration) The amplitudes of the local responses as a percentage of the g neral responses are summarized in Fig 2 where also the relative length of the stained i e the locally stimulated portion of the tissues has been indicated In the aorta (left diagram) local transmural nerve stimulation at 4—32 Hz elicited responses which were of the same magnitude relative to the general responses as the stained to the total length of the vessel The relative amplitude of the local responses to exogenous NA tended to increa e with higher NA concentrations and at 10 $^{5}$ M the percentage for the response was slightly greater than the relative length of the stained portion

The rat portal vein preparations showed typical spontaneous intermittent

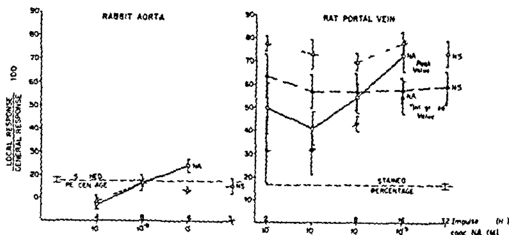



Fig 2 Local responses as a percentage of general responses to exogenous NA of graded concentrations and transmural nerve stimulations (NS) at different impulse rates for the rabbit aorta (left diagram) and the rat portal vein (right diagram) Mean values ± S E (from 4 preparations of each vessel Stained length (locally stimulated portion) expressed as a percentage of total length of preparation is indicated Maximal amplitudes during 1 min stimulation periods for aorta and portal vein peak values are shown Integrated values refer to portal vein mean force developments during respective 1 min responses obtained by integration of deflections of recording pen Note that relative magnitude of responses of the rabbit aorta correspond to the stained percentages of the preparations whereas the rat portal vein responses are significantly greater

tractions which increased in frequency and amplitude with low to moderate stimulation and tended to fuse at higher NA concentrations and nerve impulse rates The peak contraction amplitude during local nerve stimulation amounted to approximately 75 per cent of the corresponding contraction induced by general nerve activation and was independent of nerve impulse rate (Fig 2 right panel) The relative peak amplitude of the responses to local exogenous NA administration tended to increase with increasing NA concentration All the relative responses to local NA exceeded significantly the ratio of the stained to total length of the preparations

When the contractile force of the phasic portal vein responses was averaged by integration the relative mean amplitudes of the force of the responses to local nerve stimulation and to local exogenous NA administration essentially paralleled the relative peak values but were approximately 15 per cent units lower Invariably, the relative mean amplitude values were significantly greater than the fraction of stained vessel (Fig 2)

Local stimulation of the rat portal vein thus led to a relative response which was significantly greater than the relative size of the stimulated part of the preparation However in the rabbit aorta the relative amplitude of the local responses was no greater than the relative length of the stimulated portion unless very high NA concentrations were administered This difference in results from the two vessels indicates that the contractile elements in a relatively greater length of the portal vein

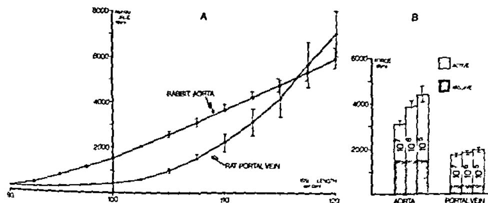



Fig 3 A Legnth tension relationships for rabbit aorta and rat portal vein preparations in $Ca^{++}$ free solution Tissue length expressed as per cent of length at the passive force 1500 and 400 dyn respectively used in previous experiments Mean±S E n = 4 B Levels of passive and active peak force during 1 min exposure to NA in concentrations indicated Same preparations as in A

had shortened and thereby produced greater force by stretch of elastic elements in the inactive part of the tissue *i e* confirmation that propagation occurs in the rat portal vein but not in the rabbit thoracic aorta

However differences in the elastic properties of the inactive parts of the tissues could partly account for these results If the force generated in the locally stimulated part of the preparations was transmitted *via* a much more rigid elastic tissue component in the portal vein than in the aorta similar results might possibly be obtained even though no longitudinal propagation had occurred This possibility was explored by constructing the length tension diagrams shown in Fig 3 A The passive elastic properties of the two vessels were studied in experiments where the passive force at different tissue lengths was recorded after 1 min stress relaxation The aortic strip showed uniform elasticity over the length interval studied whereas the portal vein preparation was much more distensible at low and moderate force levels and was more rigid at high ones In Fig 3 B the passive tension used in the previous experiments (400 and 1500 dyn) is indicated for the two vessels together with the maximum active force induced by exogenous NA (10⁻⁷—10 M) Within the range of the active fo ces recorded during local responses (20—70 per cent of these general response values) it is seen that the passive elastic properties of the two vessels are either comparable or that the rat portal vein may be less rigid It is therefore concluded that the difference between the relative magnitudes of the local responses of the portal vein and the aorta respectively (Fig 2) are due to the myogenic longitudinal spread of activity in the former and the lack of significant propagation in the latter vessel These results imply that the method adequately indicates longitudinal propagation in blood vessel preparations

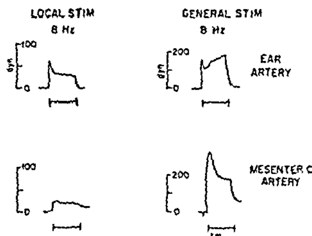



Fig 4 Isometric recordings of responses to local and general transmural field stimulation (8 Hz 0 5 ms 12 V) of intact preparations of ear artery and a mesenteric arcade artery from the rabbit Polarity of recorder inverted so that upward deflection indicates reduction in force

*Rabbit aorta muscular arteries and rat portal vein—analysis of propagation*

In order to test the ability of a given preparation to support longitudinal propagation relative values for the response to transmural nerve stimulation at 8 Hz and to exogenous NA ($10^{-5}$ M) respectively were compared to the relative length of the stained tissue portion in each group of 4 expts and tested for pair differences

The results from the *rat portal vein* and the *rabbit aorta* for transmural stimulation (8 Hz) and exogenous NA ($10^{-5}$ M) expressed in this manner are shown in

TABLE I Propagation in preparations of rabbit arteries and rat portal vein Mean±S E n = 4 Indirectly activated percentage calculated as local response (per cent) minus stained length (per cent) Statistically significant propagation (*) was concluded when the local response percentage significantly exceeded the stained percentage as tested for pair differences (p < 0 05) For further explanation see text

| Tissue | | Total length (mm) | Indirectly activated percentage | |
|---|---|---|---|---|
| | | | Nerve stimulation 8 Hz | Noradrenaline $10^{-5}$ M |
| Rat portal vein | | 13 8 ±0 63 | 53 3±5 18* | 37 8±6 8?* |
| Rabbit aorta | | 23 5 2 9? | 1 1+2 81 | −1 3±1 41 |
| Ear artery | strip | 18 0 0 ?7 | 2 0±5 79 | 10 4±2 10 |
| | intact | 18 0−1 08 | 22 0±3 54 (A)*<br>3 8±2 20 (B) | 1 8±4 87 |
| Mesenteric arcade art | strip | 20 8 0 25 | 2 5±0 76* | 7 2±1 23* |
| | intact | 18 8 1 11 | 1 8+0 63* | — |
| Brachial artery | strip | 18 ?+1 2? | −3 2±6 0? | 7 1±1 96* |
| | intact | 17 ? 0 ?? | 2 1+1 60 | — |
| Radial artery | strip | 14 3 1 60 | 0 3±3 41 | 4 1±1 99 |
| | intact | 1? ? 0 2? | 21 5±6 4 | 7 9±2 19* |
| Femoral artery | strip | 22 8 0 48 | 0 1±2 27 | 2 9±0 6?* |
| | intact | 20 0 0 82 | 1 2±2 06 | 3 5±0 34* |
| Post tibial artery | strip | 20 3 0 25 | 7 3±2 08* | 17 4±4 12 |
| | intact | 13 0±0 71 | 16 6±2 79* | 10 7±2 50 |

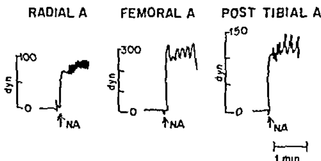



Fig 5 Isometric recording of responses to general NA ($10^{-6}$ M) administration to superfused intact preparations of the radial femoral and posterior tibial arteries of the rabbit Note superimposed rhythmical activity Polarity of recorder inverted so that upward deflection indicates reduction in force

Table I In the portal vein preparation which by necessity was shorter than the other tissues the induced activity was propagated to 26—53 per cent of the length of the preparation outside the lower chamber In contrast no propagation was found in the rabbbit aorta

In the experiments on different muscular arteries from the rabbit propagation was assessed in 4 preparations from intact vessels and 4 spirally cut strips

The response of the intact *ear artery* (o d 1 mm) to general nerve stimulation regularly displayed two distinct phases (Fig 4) similar to the biphasic NA response previously described in this vessel (Bevan and Waterson 1971) In these experiments the initial phase (A) was significantly propagated and the second one (B) was not (Table I) The responses of the intact ear artery to exogenous NA were comparatively weak and were not found to be propagated Strips of that vessel showed propagation of the responses to exogenous NA

The *mesenteric arcade artery* (o d 0 6 mm) responses to transmural stimulation also illustrated in Fig 4 were propagated to a small extent (Table I) All intact preparations were treated with cocaine $10^{-6}$ M for 30 min before exposure to exogenous NA in order to eliminate the neuronal NA uptake mechanism In spite of that treatment the intact mesenteric artery only gave small and inconsistent responses to the drug The spiral strips of this vessel responded consistently and propagation occurred during local NA administration and transmural stimulation (Table I)

The responses of the intact *brachial artery* (o d 1 2 mm) to transmural stimulation had the same general configuration as those of the mesenteric artery (Fig 4) whereas those of the strips were more sluggish and kept increasing during the 1 min stimulation period No propagation of the neurogenic responses was found The local NA responses of the strips indicated the occurrence of propagation The intact vessel did not respond consistently to exogenous NA

The induced activity of the *radial artery* (o d 0 5 mm) was rapid in onset Frequently a superimposed phasic activity was recorded in response to exogenous NA (Fig 5) Propagation of responses occurred from intact vessels but not from strips

In Fig 5 tracings from experiments on the *femoral artery* (o d 1 3 mm) and the *posterior tibial artery* at the ankle (o d 0 6 mm) are shown After rather con

siderable latency (10—15 s) the intact femoral artery contracted rapidly and rhythmic activity was regularly recorded. Significant longitudinal propagation of the local NA responses but not of the neural responses was found in both the intact preparations and the strips. The responses of the posterior tibial artery were qualitatively similar to those of the femoral artery but propagation of both NA and neural responses occurred.

In order to compare the extent of propagation in the different blood vessels an apparent distance of propagation of the individual responses was calculated in the following way: (local/general response—strained/total tissue length) × total tissue length = apparent distance of propagation. This theoretical value indicates in absolute terms the length of the tissue outside the lower chamber which would have become fully activated by propagation from the directly stimulated portion. Mean values of the apparent distances for both neurogenic and NA responses from intact as well as strip prepations of the different blood vessels are summarized in Table II. There also the results of a test for the statistical significance of the difference between the mean values are illustrated. It is seen that this measure of propagation was greatest in the portal vein and smallest (negative) in the aortic

TABLE II. Apparent distances of propagation for different blood vessels. Left column shows mean values ± S.E. calculated from experiments presented in Table I. Results of student's t test between means shown in array. P less than values indicated. For explanation see text.

| | Portal vein | Post. tibial artery | Femoral artery | Radial artery | Brachial artery | Mesenteric artery | Ear artery |
|---|---|---|---|---|---|---|---|
| Rabbit aorta<br>0.3 ± 0.33<br>n = 8 | 0.01 | 0.01 | 0.03 | 0.01 | 0.12 | 0.01 | 0.0[illegible] |
| Ear artery<br>1.C ± 0.51<br>n = 1f | 0.01 | 0.09 | 0.02 | 0.48 | 0.0[illegible] | 0.10 | |
| Mesenteric artery<br>0.8 ± 0.18<br>n = 12 | 0.01 | 0.01 | 0.07 | 0.08 | 0.[illegible]1 | | |
| Brachial artery<br>0.4 ± 0.43<br>n = 12 | 0.01 | 0.01 | 0.49 | 0.01 | | | |
| Radial artery<br>1.C ± 0.[illegible]0<br>n = 1f | 0.01 | 0.0[illegible] | 0.01 | | | | |
| Femoral artery<br>0.4 ± 0.18<br>n = 1f | 0.01 | 0.01 | | | | | |
| Post. tibial artery<br>2.5 ± 0.33<br>n = 16 | 0.01 | | | | | | |
| Rat portal vein<br>6.3 ± 0.72<br>n = 8 | | | | | | | |

strip In the rabbit muscular arteries the apparent distance tended to increase with diminution in the vascular diameter It is of particular interest that propagation in the distal limb arteries (radial and posterior tibial) was greater than in the corresponding proximal brachial and femoral arteries

## Discussion

Cell to-cell spread of activity in vertebrate smooth muscle is a process of electrical conduction due to propagation of action potentials or electrotonic spread of depolarization Detailed investigations of the passive and active electrophysiological properties of the smooth muscle of different vascular walls have been performed in some large vessels (*e g* Ito and Kuriyama 1971 Mekata 1971) but in most regions of the vascular system the membrane characteristics have yet to be studied Such a field of research is likely to present considerable technical problems in view of the size and inaccessibility of the smooth muscle tissue in the smaller blood vessels (see Holman 1968) For many purposes it may be of advantage to rely on comparisons between the functional properties of thoroughly investigated isolated vascular smooth muscle models and those from other parts of the vasculature as revealed *in vivo* or *in vitro*

The present study was prompted by the question as to what extent arterial smooth muscle can support longitudinal spread of activity

The experimental design was based on the contention that active force developed by the isolated blood vessel preparations is due to a contractile element (CE) stretching a series elastic element (SE) and that the passive tension of inactive tissue is supported by a third parallel elastic element (PE) When only part of the tissue length is activated the relative amount of internal CE shortening will be expected to be proportional to the relative length of the activated portion of the preparation In such a situation the locally activated CE will be in series with SE within the active part and with PE in the inactive part of the tissue If the compliance per unit length of SE and PE were comparable the isometric force generated when CE shortened in only one part of the tissue would be related to the force when the entire preparation was stimulated as the locally activated to the total tissue lengths Contractile activity in tissue parts beyond the directly stimulated length of the preparation would then manifest itself as a local response of greater relative magnitude than that of the relative length of the stimulated portion

The method was empirically tested in experiments on the rat portal vein preparation where longitudinal myogenic propagation has been shown to occur (Johansson and Ljung 1967 Ljung and Stage 1970) and on the rabbit thoracic aorta where propagation is considered insignificant (Bevan *et al* 1970) It was found that the relative amplitude of all contractile responses to localized stimulation significantly exceeded the relative length of the locally stimulated tissue in the experiment on the rat portal vein but not in those on the rabbit thoracic aorta (Fig 2 Table 1

Since only 20 per cent of the preparations were directly activated it was of interest as to whether differences in the characteristics of PE in the inactive parts of these vessels could markedly influence the relative magnitude of their respective local responses As seen in Fig 3, PE in the inactive portal vein preparation was if anything more compliant than in the aortic strip within the relevant force interval Thus it can be assumed that the relative length of the activated tissue (directly and *via* propagation) *i e* the amount of internal CE shortening mainly determined the relative amplitude of the local response and that variations in elastic properties were of minor importance Therefore we conclude that the results show the occurrence of propagation in the rat portal vein and the lack of it in the aorta and that the method is suitable for the purpose of this study

It was crucial to determine the length of the tissue which became locally stimulated under these experimental conditions Local transmural nerve stimulation was performed using the horizontal electrodes on either side of the tissue in the lower chamber It is not likely that an effective electrical current field during stimulation could extend beyond the lucite chamber Of course some intramural axons with terminals in the upper part of the tissue might have become excited by the local field However the transverse electrode arrangement would theoretically reduce the probability of inducing propagated action potentials in longitudinal axons (see Ranck 1966)

Exogenous NA was administered to the lower chamber by perfusion after the slit in the eparation had been sealed with grease (see Fig 1) The length of the preparation which became stained when Evans blue was added to the perfusate was taken to represent the exposed part of the tissue The drug might have diffused within the tissue above the separation and the superfusate may have decreased the NA concentration below Such interaction between the superfusate and the perfusate might explain the increase in the relative amplitude of the responses to exogenous NA with increase in drug concentration found in both the rabbit aorta and the rat portal vein It is conceivable that the absolute distance of electrical conduction might have increased when a stronger excitation was induced (Burnstock and Prosser 1960) Apparently this is of small mechanical significance in these experiments since the relative responses to local stimulation at different nerve impulse rates remained essentially unchanged (Fig 2)

In the rabbit muscular arteries tested propagation increased towards the periphery of the vascular system The apparent propagation in some experiments on the distal limb vessels approached that in the rat portal vein The rhythmicity of the responses of the intact femoral radial and posterior tibial arteries to exogenous NA also demonstrate the existence of a mechanism for intercellular coordination The morphological and electrophysiological characteristics of these rabbit vessels are not known Intercellular low resistance connections (nexuses) have been found in several different blood vessels (see Somlyo and Somlyo 1968) and have been shown to increase in number with decreasing diameter of resistance vessels (Rhodin 1967)

The passive electrical properties can provide an indication of the number and closeness of cell to-cell connections (Tomita 1970) In two recent reports it was demonstrated that the smooth muscle membrane of the guinea pig portal vein (Ito and Kuriyama 1971) and the rabbit common carotid artery (Mekata 1971) showed cable like properties In the carotid artery an elastic vessel with electrophysiological features similar to the major elastic arteries of the rabbit (Su, Bevan and Ursillo 1964 Mekata and Niu 1972) the space constant is actually greater than in the portal vein Since we found no propagation in the rabbit aorta and considerable spread in the rat portal vein it is obvious that the passive electrical properties as determined by the cell membrane characteristics and the inter cell relations in different blodo vessels do not necessarily parallel the extent of propagation of locally induced activity Although nexuses are of course a prerequisite for myogenic conduction it seems that other properties of the smooth muscle may largely determine the functional importance of propagation in the response of a particular vessel

There is only limited information available about the electrophysiology of the smooth muscle in muscular arteries Speden (1967) measured membrane potential changes *in vitro* in rabbit ear and small mesenteric arteries No spike like action potentials were recorded in response to nerve stimulation but only excitatory junction potentials and graded sustained depolarization In similar vessels studied *in vivo* in the guinea pig action potentials were recorded (Speden 1964) Subsequently it was concluded that the e potentials may represent non propagative or rapidly decremental activity (Speden 1970) In small mesenteric arteries and arterioles from the rat Steedman (1963) recorded spontaneous slow wave potentials and action potentials, which resemble the propagated electrical activity of longitudinal intestinal muscle (Holman 1968)

If a minimum cross sectional bundle of smooth muscle is needed to support regenerative electrical spike activity in vascular as well as in other smooth muscle (see Bennet and Burnstock 1968) it might be expected that propagative action potential activity would become less likely in the vessel wall with a small radius The fact that in our experiments propagation of activity increased towards the periphery favours the possibility that there might be a basis for propagation other than regenerative action potentials spreading along a smooth muscle bundle in the rabbit arterial tree It may well be that a coupled slow wave activity is important for spread of activity in these blood vessels (Bevan and Su 1973)

It is of interest that the initial but not the second phase of the bimodal response of the ear artery was found to be propagated which confirms an earlier suggestion (Bevan and Waterson 1971) Subsequent studies have shown that two mechanisms of activation can be recruited in this vessel by NA (Bevan *et la* 1973)

The apparent distances of propagation were calculated mainly to provide a basis for comparison of an absolute rather than a relative value of propagation in the different vessels They would be expected to considerably underrate the actual distance that some activity spread along the vessels In a previous electrophysiological study (Ljung and Stage 1970) it was seen that the portal vein s

activity, which is associated with bursts of propagated action potentials often originates from one single pacemaker region to synchronize the entire preparation During topical NA application the locally induced bursts of activity undoubtedly spread along the whole length of the preparation, but decreased in duration with distance from the site of stimulation Therefore the mechanical response induced in one part of the portal vein by myogenic propagation of activity from another stimulated portion will be comparatively small in amplitude (Johansson and Ljung 1968) Such an explanation accounts for the present finding that the apparent distance of propagation for the peak response in the portal vein amounted to 50—70 per cent of the tissue length outside the lower chamber although spread of electrical activity can be expected along the entire preparation

The calculated distances of propagation also have to be judged in the light of the altered conditions involved in any *in vitro* experiment In the rat aorta Biamino and Kruckenberg (1969) found longitudinal propagation only when the potassium ion concentration was raised or vasoactive agents were added to the Tyrode solution It is quite possible that factors such as adequate distension and nervous and humoral influences will favour propagation in the rabbit vessels *in vivo*

However even with due consideration to the approximations involved in the present assessment of propagation the limited extent of conduction found in the muscular arteries indicates that myogenic propagation is important mainly for local spread and coordination of activity within the vascular wall

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# The Neuromuscular Blocking Action of an Isolated Toxin from the Elapid (Oxyuranus scutellactus)

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## Abstract

KAMENSKAYA M and S THESLEFF *The neuromuscular blocking action of an isolated toxin from the Elapid (Oxyuranus scutellactus)* Acta physiol scand 1974 *90* 716—724

Intravenous administration of the neurotoxin (taipoxin) to mice caused after a latent period death by respiratory paralysis The length of the latent period varied with dose the dose response relation being bilogarithmic Taipoxin failed to affect the resting membrane potential and the action potential of muscle It caused a slight reduction in the acetylcholine sensitivity of the muscle membrane The main effect of taipoxin on neuromuscular transmission was to reduce spontaneous and evoked transmitter release The frequency of spontaneous miniature end plate potentials (m.e p p.s) was reduced to complete stop and depolarization of nerve terminals by a high potassium concentration (20 mM) failed to cause a marked and sustained increase in m e p p frequency With nerve stimulation the amplitude of end plate potentials (e p p s was reduced and with repetitive stimulation the amplitude of e p p.s rapidly fell to a low level Post tetanic facilitation of m e p p frequency and of e p p amplitude were absent The shape of evoked e p p s indicated asynchronous release of transmitter quanta The effects of the toxin on neuromuscular transmission progressed even when the muscle was removed from the animal and placed in toxin free bathing fluid *In vitro* experiments showed that the rate of onset of neuromuscular block depended on the temperature of the bathing solution and on the degree of activity at the neuromuscular junction Thus incubation in toxin solution during 4 hr at 28 C failed to produce a neuromuscular block while at 37 C the block was complete within 1 h With nerve stimulation at 10 Hz for 2 s every 2 min the rate of blockade was more rapid than in unstimulated preparations similarly 20 mM potassium in the bathing fluid enhanced the rate of blockade

Venoms of snakes belonging to the families *Elapidae* are highly toxic and act by producing flaccid paralysis and respiratory failure in animals These effects are attributable to the so-called neurotoxins present in these venoms The neurotoxins can be classified into two groups depending upon their mode of action One group of neurotoxins produce neuromuscular block by a curare like action on cholinergic receptors while the other group acts presynaptically on motor nerve endings (Lee 1972)

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Recently Fohlman *et al* (1973) isolated a neurotoxin from the Australian *Elapidae* Taipan (*Oxyuranus scutellactus*) which in preliminary tests proved highly toxic This neurotoxin subsequently called taipoxin is a complex between a glycoprotein $\alpha$ component and a carbohydrate free $\beta$ component which are separable by gel filtration at low pH The molecular weight of the $\beta$-component is about 14 000 but the molecular weights of the $\alpha$ component and of the intact complex have not been unambiguously established

*In the present* study we have tried to elucidate the mode of action of taipoxin on mammalian neuromuscular transmission The results show that this neurotoxin blocks neuromuscular transmission by acting presynaptically on motor nerve terminals blocking spontaneous and evoked transmitter release

## Methods

The experiments were made on male NMRI mice weighing 25—30 g Taipoxin 1—2 *ug* in 0 1 ml 0 9% NaCl solution was injected into the tail vein of the animal In a few experiments were the $\alpha$ or $\beta$ fraction of taipoxin injected i v The phrenic nerve hemidiaphragm *muscle (in some instances the extensor digitorum longus muscle)* was *dissected out* either when the animal showed the first signs of neuromuscular weakness (early stage of paralysis) or when respiratory paralysis occurred (late stage of paralysis) The nerve muscle preparation was mounted in a constant flow bath which was continuously perfused with an oxygenated fluid (Liley 1956) maintained at pH 7 2—7 4 and at 28 C In some experiments taipoxin 1 *ug*/ml was added to an organ bath containing a previously unpoisoned nerve hemidiaphragm preparation The phrenic nerve was stimulated by bipolar platinum electrodes *using square wave pulses of supramaximal voltage and 0 5 ms duration*

Conventional intracellular recording techniques were used for recording of resting membrane potential miniature end plate potentials and end plate potentials The acetylcholine sensitivity of chronically denervated muscles was assessed by the technique of iontophoretic micro-application (del Castillo and Katz 1955) Action potentials were generated and recorded using a double micro-electrode technique (Redfern and Thesleff 1971) The responses obtained were recorded on a polygraph (Mingograph 81) and/or photographed from the oscilloscope trace

## Results

*Effects of taipoxin given intravenously*

With i v administration was the median $LD_{50}$ *dose of taipoxin about 5 µg/kg* but the animal dying of respiratory arrest and flaccid paralysis Upon injection of a lethal dose a latent period was observed before paralyses The length of the latent period varied with dose as shown by the survival time in Fig 1 and appeared to be a logarithmic function of toxin dose also expressed logarithmically With higher doses ($50$—$200 \times LD_{50}$) the survival time was little further affected indicating tissue saturation and that a minimal time of 20—30 min is required for the toxin to cause respiratory paralysis

In muscles from animals dying from respiratory paralysis the resting membrane potential was normal as shown by the values in Table I Similarly the table shows that the toxin failed to affect the rate of rise the threshold potential for spike generation and the amount of overshoot of the action potential in single muscle fibres In mice with chronically denervated extensor digitorum longus (EDL

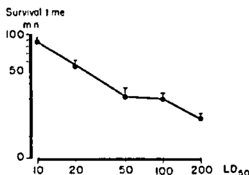



Fig. 1

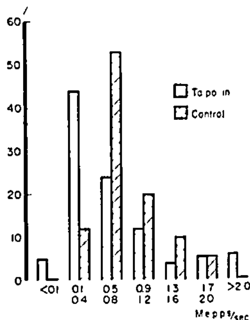



Fig. 2

Fig. 1. Relation of dose expressed as multiples of mean LD50 of taipoxin to mean ± S.D. (5 animals) of survival time of mice.

Fig. 2. Frequency distribution of m.e.p.p.s expressed as per cent of 76 fibres from taipoxin poisoned muscles and of 40 fibres from unpoisoned control diaphragm muscles.

muscles taipoxin had a small effect on the sensitivity of the muscle membrane to micro-iontophoretically administered acetylcholine, the sensitivity being reduced from an average of 43 mV/nC to 28 mV/nC (Table II).

The observation that taipoxin had little effect on the excitability of skeletal muscle suggested that its major site of action was on the motor nerve and transmitter release; this parameter was therefore investigated in more detail.

*Spontaneous transmitter release*

In the early stage of paralysis spontaneous miniature end plate potentials (m.e.p.p.s) were recorded in most of the muscle fibres but later only in a small portion of them. The values presented in Table III show that in the fibres with m.e.p.p.s their mean frequency was close to the value observed in untreated control muscles. However, a study of the frequency distribution, as shown in Fig. 2, indicated a shift towards a reduction in frequency and a wider frequency distribution than in control muscles. In many fibres spontaneous bursts of m.e.p.p.s at a higher frequency were recorded, the m.e.p.p. frequency always being reduced following the end of the burst as shown by the graph in Fig. 3. These bursts often preceded a complete and abrupt stop of transmitter release.

TABLE I Resting membrane potential (RMP), rate of rise of action potential, threshold potential for spike generation and overshoot of the action potential in EDL muscles of normal mice (control) and of mice during the late stage of paralysis following i.v. administration of taipoxin 1.5 μg. The values are means ± S.E. and the figures within parentheses give number of fibres examined.

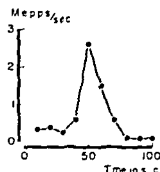
| Treatment | RMP mV | Rate of rise V/sec | Threshold mV | Overshoot mV |
|---|---|---|---|---|
| Control | 79 ± 0.7 (31) | 589 ± 23.5 (32) | 54 ± 0.5 (32) | 43 ± 0.5 (32) |
| Taipoxin 1.5 μg i.v. | 81 ± 0.5 (21) | 552 ± 22.5 (21) | 54 ± 0.5 (21) | 41 ± 0.9 (21) |

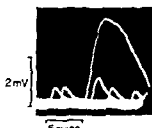
TABLE II The ACh sensitivity and resting membrane potential (RMP) of EDL muscles denervated for 6 days in untreated animals (controls) and in animals during the late stage of paralysis following i.v. administration of taipoxin 1.5 μg. The ACh sensitivity was determined by micro-iontophoretic application of ACh to extrajunctional membrane areas and is expressed as mV depolarization in response to $10^{-9}$ Coulomb passed through the ACh pipette. The values are means ± S.E. and the figure within parenthesis denotes the number of fibres examined.

| Treatment | RMP mV | ACh sensitivity mV/nC |
|---|---|---|
| Control | 57 ± 1.3 (13) | 43 ± 3.7 (13) |
| Taipoxin 1.5 μg i.v. | 56 ± 1.1 (17) | 28 ± 2.4 (17) |

TABLE III The effect of taipoxin and its α and β fraction on the frequency of m.e.p.p. in normal bathing fluid and in the presence of 20 mM $K^+$. The values are means ± S.E. The figure within parentheses denotes the number of fibres examined.

| | M.e.p.p. frequency/sec | M.e.p.p. frequency /sec in 20 mM $K^+$ |
|---|---|---|
| Control | 0.9 ± 0.08 (40) | 114.3 ± 5.78 (5) |
| Taipoxin 1 μg i.v. | 0.8 ± 0.11 (76) | 1.2 ± 0.22 (9) |
| β taipoxin 10–20 μg i.v. | 0.5 ± 0.08 (36) | 5.3 ± 4.3 (23) |
| α taipoxin 100 μg i.v. | 0.4 ± 0.09 (10) | 1.1 ± 0.44 (5) |



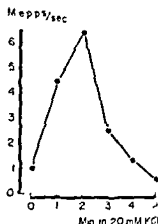




Fig 3 Fig 4 Fig 5

Fig 3 Spontaneous acceleration of m e p p frequency in a single fibre of a taipoxin poisoned muscle Note a reduction of m e p p frequency following the end of the burst

Fig 4 Superimposed tracings of spontaneous m e p p s showing one gigant m e p p with notches on its falling phase

Fig 5 The effect on the frequency of m e p p s in a fibre of raising the potassium concentration of the bathing fluid from 5 to 20 mM The high potassium concentration was introduced into the organ bath at zero time 1 min being required for a complete change of solution

Frequently the amplitude and the duration of a spontaneous m e p p was increased or the potential had notches on its rising or falling phase indicating an asynchronous release of two or more quanta of transmitter (Fig 4)

Of interest was that irrespective of at which stage of paralysis the muscle was removed from the animal the effect of the toxin progressed in the toxin free bath solution the m e p p frequency being reduced with the time until complete stop

As shown by Table III a high potassium concentration (20 mM) in the bathing fluid failed to produce a marked and sustained increase in the mean rate of transmitter release in poisoned muscle while it in control muscles altered the frequency from 0 9 to 114/s In some fibres however the elevation of the potassium concentration caused a shortlasting increase of transmitter release followed by depression as shown in Fig 5 Elevation of the external calcium concentration from 2 mM up to 16 mM or the addition of guanidin 5 mM and produced only a small and always a shortlasting increase in the frequency of m e p p s in response to high potassium showing that the normal depolarization transmitter secretion coupling in the nerve terminals was severely and apparently irreversibly impaired by the toxin

*Evoked transmitter release*

In muscles removed during the early stage of paralysis nerve stimulation evoked action potentials and mechanical twitches in single fibres while later only end plate potentials (e p p s) could be recorded It was therefore possible to study evoked transmitter release in normal bathing solution without the use of special blocking agents

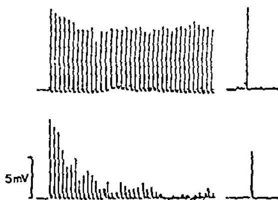



Fig 6 Lower record shows the fall in e p p amplitude down to the size of single quanta, during a train of nerve stimuli at 50 Hz in a taipoxin poisoned muscle 2 s after the end of train a single shock gave a large e p p showing recovery from depression Upper record is from an unpoisoned muscle showing that a similar train of nerve impulses has relatively little effect on e p p amplitude In this muscle neuromuscular transmission was blocked following glycerol treatment

During the early stage of paralysis a train of nerve stimuli initiated action potentials in the very beginning of the train but rapidly the amplitude of the e p p.s fell below the threshold of the fibre resulting in neuromuscular block The higher the frequency of stimulation the more marked was the fall in e p p amplitude Fig 6 shows the fall in e p p amplitude in response to nerve stimulation at 50 Hz in a late stage of paralysis the figure including for comparison (upper record) e p p s in an unpoisoned muscle The run down was reversible as shown by the test pulse a few seconds after the end of the train It was of interest that in spite of an initial relatively low e p p amplitude repetitive stimulation rapidly reduced e.p p size to that of single quanta

Typical of a taipoxin poisoned muscle was that the e p p.s frequently fluctuated in size and in duration the nerve impulse causing an asynchronous release of quanta leading to split e p p s as shown in Fig 7

In contrast to normal muscle post tetanic facilitation of m e p p frequency was not present and in fact many times the m e p p frequency was decreased following a period of high frequency stimulation Post tetanic potentiation of e p p.s was also absent.

The presence of 4—16 mM calcium or of 5 mM guanidine in the bathing solution markedly increased the amount of transmitter released but in poisoned muscles only during the first impulses of a train

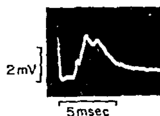



Fig 7 Typical e p p in a single fibre from a taipoxin poisoned muscle showing asynchronous release of quanta leading to a "split and prolonged e p p

As previously mentioned the blocking action of the toxin progressed while the preparation was in the bathing fluid and after a few hours the nerve failed to release transmitter.

I.v. administration of 20—100 μg of either the α or of the β fraction of taipoxin produced a progressing flaccid paralysis and a study of neuromuscular transmission revealed that both compounds affected spontaneous and evoked transmitter release in a qualitatively similar way as the native taipoxin (see Table III), the only apparent difference being a lower potency.

*Effect of taipoxin in vitro*

The addition of taipoxin 1 μg/ml to an organ bath containing a normal phrenic nerve hemidiaphragm preparation affected neuromuscular transmission in a way qualitatively similar to that observed following *in vivo* administration. Spontaneous and evoked transmitter release were affected in the same way as already described. Enhancement of spontaneous transmitter release was generally not encountered during any stage of toxin action but occasional spontaneous bursts of m.e.p.p.s at increased frequency were recorded as has been described for *in vivo* poisoned muscles.

Of interest was that the rate of onset of neuromuscular blockade depended on the temperature of the bathing solution and on the degree of activity at the neuromuscular junction. Thus incubation in toxin solution during 4 h at 28° C failed to produce a neuromuscular block while at 37° C the block was complete within 1 h. With nerve stimulation at 10 Hz for 2 s every 2 min the rate of blockade was more rapid than in unstimulated preparations. Similarly taipoxin acted more rapidly when 20 mM potassium was in the bathing fluid. A quantitative report of these effects will appear elsewhere.

The effects of taipoxin were irreversible; after 1.5 h in toxin solution 1 μg/ml at 37° C no recovery of neuromuscular transmission occurred even after 6 h of washing in toxin free bathing fluid.

## Discussion

The results of the present study show that taipoxin acts presynaptically blocking transmitter release from motor nerve terminals. The exact mode by which taipoxin exerts its action is not clear but the observation of a reduction of spontaneous and evoked transmitter release, the lack of nerve terminal depolarization in promoting release, the lack of post tetanic potentiation of release and the appearance of split m.e.p.p.s and e.p.p.s all suggest that either quantal formation and/or the quantal release process are the main targets for the toxin. In most of these respects taipoxin acts in a way similar to β bungarotoxin of *Bungarus multicinctus* (Lee and Chang 1966) and to the neurotoxin (notexin) from the Australian tiger snake (*Notechis scutatus scutatus*) as shown by Harris, Karlsson and Thesleff (1973). It should also be mentioned that botulinum toxin appears to have this mode of action (Burgen

1972) and that its dose—survival time curve like that of taipoxin is bilogarithmic (Simpson 1971). In contrast to taipoxin which is moderately acidic (I P 5 0) notexin is a very basic protein with a mol wt of 13 574 (Karlsson Eaker and Ryden 1972). The integral amino acid compositions of β bungarotoxin and botulinum toxin have not been published but the mol wt. of the former has been estimated at 28,500 (Lee 1973) and that of botulinum toxin at about 60 000 (Burgen 1972). Thus it appears that polypeptides with widely differing chemical properties and size may in a similar way interfere with transmitter release. The mode of action on the release process may however on the molecular level differ between these toxins. For instance in contrast to notexin (Kamenskaya unpublished) and taipoxin β bungarotoxin initially markedly increases the frequency of spontaneous m.e p p.s (Lee and Change 1966) and with repetitive stimulation the amplitude of e p p.s is sustained (Lee 1972).

On the other hand the mode of action of these toxins share several interesting and unusual features. They are highly potent and apparently have a selective affinity for cholinergic nerves. Poisoning occurs after a latent period inversely related to toxin dose while an irreversible binding of the toxins to the tissue seems to be almost complete during the latent period. The poisoning has a high temperature coefficient and the time course of the paralysis is markedly accelerated by procedures leading to increased transmitter release *i.e.* by nerve stimulation and by high external potassium concentrations (Lee 1970 Harris *et al* 1973).

As a possible explanation for these common features we tentatively suggest that the toxins are irreversibly fixed to membrane sites in nerve terminals the number of molecules bound being proportional to toxin dose. Thereafter a time-consuming energy requiring and of dose independent mechanism for instance pinocytosis transfers toxin molecules to the inside of the terminal where the toxin interferes with quantal formation and/or release. This sequence would explain the bilogarithmic dose response curve of survival time versus toxin concentration the observation of a minimal period before paralysis and the temperature dependence of action. The observed dependence of onset of paralysis upon preceeding transmitter release suggests that the uptake process is linked to the turnover of transmitter speculatively to the recycling of membrane accompanying transmitter release Heuser 1971 Heuser and Reese 1972

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Acta physiol scand 1974 90 725—730

From the Laboratory for the Theory of Gymnastics University of Copenhagen Denmark

# Effects of Changes in Plasma Volume and Osmolarity on Thermoregulation during Exercise

By

BODIL NIELSEN

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## Abstract

NIELSEN B *Effects of changes in plasma volume and osmolarity on thermoregulation during exercise* Acta physiol scand 1974 *90* 725—730

Plateau values of deep body temperature were diminished during work after intake of 1¾ l water they were increased after intake of 2—3 % sodium chloride solution and after dehydration in a sauna by 1.2 kg The shifts in plateau values were related to plasma osmolarity not to plasma volume In rest and during work at low temperature (7 °C) when there was virtually no sweating the effect of plasma osmolarity on body temperature disappeared The rate of increase in sweating at the start of work was faster than normal after intake of water and slower after intake of sodium chloride solution and dehydration The mechanism by which the changes in temperature were brought about is the increase in sweating at the start of work Plasma osmolartiy may cause these changes either by a peripheral effect on the sweat glands or a central effect on the thermoregulatory centres

Deep body temperature increases during exercise rising to a steady plateau level which is maintained for the duration of the exercise (M Nielsen 1938) The extent of this increase is affected by the water balance of the subjects the increase is greater in dehydrated subjects while hyperhydration reduces it (Pitts *et al* 1944 Ladell 1955 Moroff and Bass 1965 Ekblom *et al* 1970 and Greenleaf *et al* 1971) Nielsen *et al* 1971 showed that the effect of the state of hydration on body temperature during work is related to plasma osmolarity rather than to plasma volume

The aim of the study reported here was to repeat the experiments at high and low temperature *i e* in conditions with high and low activity of the sweating mechanism This was done to clarify whether plasma osmolarity acted peripherally on the sweat gland or centrally by shifting the set point of the temperature center in the hypothalamus

## Methods

Deep esophageal temperature ($T_{es}$) was measured with a thermocouple inserted through the nose and placed in the lowe esophagus just above the diaphragm (Nielsen and Nielsen 1962) The placing was controlled in each subject by X ray photography The temperature w. recorded on a Kipp Micrograph BD 1

The skin temperature ($T_s$) was measured at 15 locations with a skin thermocouple (Nielsen and Nielsen 1965). The average skin temperature ($\bar{T}_s$) was calculated by weighting each measurement according to the size of the corresponding skin area (Hardy and DuBois 1938).

The measurements of esophageal temperature were accurate to within ±0.025 C, skin temperature measurements to ±0.05 C.

The evaporation of sweat was measured as weight loss recorded continuously on a Krogh balance (Krogh and Trolle 1936) and was corrected for respiratory weight loss due to evaporation and gas exchange.

The respiratory gas exchange was measured by the Douglas bag method and air samples were analyzed in duplicate according to Scholander (1947).

Conductance of the peripheral tissues (K) (Burton 1934, Hardy 1937, Winslow *et al.* 1937) was calculated for the last 15 min of work

45—60 min as

$$K = \frac{H - E_l - S}{(T_{es} - \bar{T}_s)\,A_D}\ \text{Kcal/h m}^2\ \text{C}$$

Where H = total heat production = M − W

M = (oxygen uptake l/min × 4.9 × 60 kcal/h

W = work rate (Kpm/min × 60 × $\frac{1}{427}$ kcal/h

$E_l$ = evaporative heat loss in the lungs

S = storage of body heat $\Delta$ ($\bar{T}_e$ × 0.65 + $\bar{T}_s$ × 0.35) × body weight × (specific heat of the body)

$A_D$ = DuBois area m

Blood samples (5 ml) were drawn from a cubital vein before and after the 60 min work carefully avoiding stasis. Two hematocrit tubes were filled and they and the remaining blood samples were centrifuged for 30 min at 3000 rev/min. The plasma was removed and stored in a refrigerator. The hematocrit was expressed as the average of the two samples. Plasma osmolarity was measured by a freezing point depression osmometer* (Advanced L3) to ± 1 mOsmol/kg. Double analyses on each sample were checked against standard 100 and 500 mOsmol/kg solutions.

The exercise was done on a Krogh bicycle ergometer suspended in the Krogh balance as described previously (Nielsen and Nielsen 1965). The experiments took place in a climatic chamber at 7 ±1/4 C and at 30 ±1/4 C ambient temperature without forced air movement. Humidity was maintained below 75 % relative humidity.

In random order the subjects worked in normal condition after they had taken 1½ l water at body temperature to give hyperhydration after drinking 1½ l 2—3 NaCl solution or after sweating in a sauna to give dehydration. There was 1—2 days interval between experiments on the same subject.

## Procedure

In preliminary experiments after the subjects had taken 1 l 2 % NaCl p.o. the hematocrit and plasma osmolarity were followed for 4—7 h. Since the maximum blood volume (minimum hematocrit) and plasma osmolarity were reached at 2—4 h the work experiment was started 2—3 h after the subject drank NaCl solution. Since water is cleared rapidly the subjects started to work 1/2 h after drinking water and in some experiments ADH** (5 IU) was sprayed twice into the nose to prolong plasma hyperhydration 2—3 h or the evening before the experiment dehydration was produced by repeated exposure to heat in a sauna until the weight loss amounted to about 1½ kg. No fluid was taken from dehydration till after the experiment.

The subjects were fasting. After inserting the esophageal thermometer they rested on a cot for 45 min at 20 C covered with a blanket. T was then measured, the first blood sample drawn, and the subjects worked in the climatic chamber at a rate corresponding to about 45 of their maximum V (estimated from heart rate) for 60 min. This was in all experiments 720 kpm/min for ON and 900 kpm/min for NF. Heart rate was counted from an ECG recording every 5 min. Metabolic rate was measured from Douglas Bags filled at 30 and 40 min and $\bar{T}$ and the temperature of air and walls were measured at 35, 45 and 55 min. At the end of work the second blood sample was drawn.

* Obtained through a grant from the Danish Natural Science Research Council.

** Vasopressin spray Sandoz 1 ml 0 IU Lysinovasopressin synth.

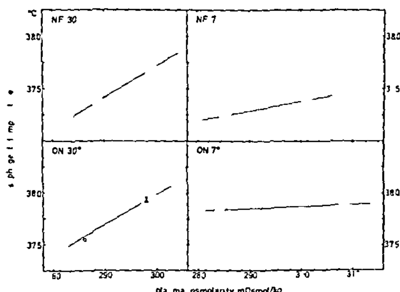



Fig 1 Plateau values of esophageal temperature plotted against final plasma osmolarity in 4 conditions ● normal O hyperhydration with water △ after drinking NaCl solution + dehydration Ambient temperature 30 C and 7 C Two subjects NF and ON

## Results

Data on the subjects and number of experiments are presented in Table I They were well trained students of physical education Two subjects worked at high and low ambient temperature in their normal state after drinking water or 2—3 % NaCl solution and during dehydration Plateau body temperature during work was related to osmolarity in agreement with earlier findings (Nielsen *et al* 1971) but only at high ambient temperature (30° C Fig 1) At low ambient temperature the relation virtually disappeared (Fig 1) The same reduced response at low temperature was found in preliminary experiments in a third subject ($\bar{T}$ normal 37 62 NaCl 37 73 and 37 55 water 37 47 Plasma osmolarity was not measured)

The average skin temperature $\bar{T}_s$ increased with increasing plasma osmolarity (Fig 2) The increase was most pronounced at 7 T in subject NF at 30 T

Table I Data on subject and number of experiments in different condition

| | height cm | weight kg | age years | T | normal | NaCl −1 ? 1 | H O −1 1 | Sauna −1 kg |
|---|---|---|---|---|---|---|---|---|
| NF | 187 | 88 | 19 | 30 C | 3 | 3 | 2 | 2 |
| | | | | C | 3 | 4 | | ? |
| ON | 183 | 0 | ?? | 30 C | 4 | 3 | 3 | 2 |
| | | | | C | 4 | 4 | | ? |
| KH | 179 | 0 | 21 | 7 C | 1 | ? | | 0 |

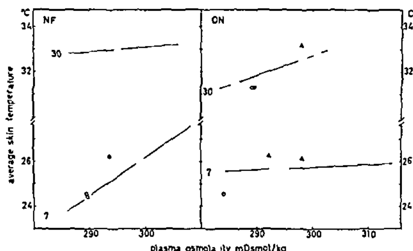



Fig 2 Average skin temperature plotted against final plasma osmolarity in 4 conditions ● normal O hyperhydration with water △ after drinking NaCl solution + dehydration Ambient temperature 30 C and 7 C Two subjects NF and ON

in subject ON The rate of sweating in the steady state Sw was slightly reduced in the sauna and the NaCl experiments at low ambient temperature (Fig 3) The reason for discrepancies between changes in Sw and change in $\bar{T}_s$ with plasma osmolarity e g ON 30° C (Fig 2 and 3) is probably, that steady state in heat balance has not been reached completely in the 60 min experiment

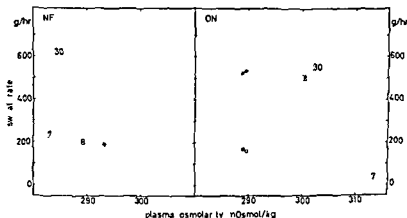



Fig 3 Steady state values of sweat rate versus final plasma osmolarity in 4 conditions ● normal O hyperhydration with water △ after drinking NaCl solution + dehydration Ambient temperature 30 C and 7 C Two subjects NF and ON

Sweat rate as indicated by loss of weight during work increased faster to a steady state when water than when NaCl solution was ingested (Fig 4) There was no consistent relation of heart rate and conductance to plasma osmolarity

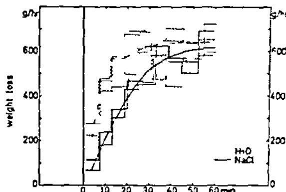



Fig 4 Rate of weight loss during 60 min work after drinking water (3 expts ) and after drinking NaCl solution —— (2 expts ) Subject ON $T_a$ 30 C

## Discussion

The results of the present study are in agreement with our earlier findings (Nielsen et al 1971) that the level of body temperature reached during exercise is related to plasma osmolarity when plasma osmolarity is increased by intake of 2 or 3 % NaCl solution or by dehydration the level of temperature is higher than when plasma osmolarity is decreased by intake of water

The shift in level is not related to circulatory factors Cardiac output was nearly identical in all the experimental conditions (Nielsen et al 1971) Even during quite severe dehydration (5 %) cardiac output during submaximal and maximal work is unchanged (Saltin 1964) Nor was there a relation of plateau temperature and plasma volume (measured by changes in plasma colloid osmotic pressure or by hematocrit) (Nielsen et al 1971 and present study)

Snellen et al (1972 a and b) found that the effect on heat elimination of drinking water or hypertonic saline could be subdivided into 1) a change due to the temperature of the fluid drunk 2) a change due to the volume drunk and 3) an additional change due to the osmolarity change produced in the body In their relative few experiments with hypertonic solutions the effect of osmolarity could not be estimated quantitatively In our experiments (1971 and present study) the subjects drank hypertonic saline several hours before the work test and the water was taken at body temperature so the effect found cannot have been due to the immediate reactions to drinking observed by Snellen et al (1972 a and b) Since the volume of saline and water drunk was the same whereas the effect was opposite volume is ruled out as an explanation for the findings reported here

The possible mechanism by which differences in plateau temperatures are obtained is illustrated by Fig 4 It was found that the initial rate of increase in sweating was influenced by the plasma osmolarity When hypertonic saline was taken sweating increased at a lower rate more heat was accumulated and consequently a higher equilibrium body temperature is attained while sweating increased sooner and levelled off at a slightly higher rate after intake of water Therefore less heat was accumulated and plateau body temperature became lower when the plasma osmolarity was reduced

Values from normal condition not shown in the graph, fall between the two curves At low temperature and at rest, when sweating was nearly absent, virtually no effect of plasma osmolarity was found (Fig 1)

The precise mechanism by which osmolarity changes affect sweat gland function is not known

Hyperosmolarity may act peripherally by reducing the sensitivity of the sweat glands to their neural drive, or by interfering with the secretion absorption processes Or the effect could be on the activity in the temperature centers in the brain *i e* changing its gain so that its temperature has to be higher for its output to the sweat glands to be the same as in normal condition Alternatively the set point in the brain centers could have been shifted (Mitchell *et al* 1971)

The proposal that the set point is changed like in fever seems unlikely because in that case the effect should also have been found at rest and in the cold whereas only small and inconsistent changes was found in these conditions

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Acta physiol scand 1974 90 731—742

From the Laboratory for the Theory of Gymnastics University of Copenhagen Denmark

# Lactate Production and Anaerobic Work Capacity after Prolonged Exercise

By

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## Abstract

Asmussen E K Klausen L E Nielsen O S A Techow and P J Tønder. *Lactate production and anaerobic work capacity after prolonged exercise* Acta physiol scand 1974 *90* 731—742

It has been found that previous heavy or long lasting exercise results in a diminished ability to raise the blood lactate concentration by exhaustive supermaximal work (Hedman 1957 Åstrand *et al* 1963 and others) No definitive explanation for this phenomenon has been given It was the purpose of the present experiments to throw light on the question whether a diminishing store of carbohydrates in the body could be the cause for the declining ability to liberate energy anaerobically by breakdown of glycogen to lactic acid In 3 subjects the stores of glycogen were presumed to be varied 1) by repeated bouts of supermaximal and submaximal exercise on the bicycle-ergometer and 2) by diet after previously depleting the carbohydrate stores by exercise It was found that in all cases the ability to work anaerobically and to produce lactate decreased with decreasing amounts of available carbohydrate Disturbances in water or electrolyte balance were avoided It is tentatively suggested that the velocity of the enzymatic process glycogen—glucose 1 phosphate is the limiting factor and that this process is slowed down by lactic acid accumulation the more the lower the substrate (glycogen) concentration is Glucose which enters the glycogenolytic chain as glucose 6-phosphate can partly restore the ability for lactate production


R. Hedman (1957) observed that the blood lactate concentration in participants in long-distance ski races (30—42 km) never exceeded 20 mg % (2 2 mM) at the end of the race In a later study Åstrand *et al* (1963) found that the maximal blood lactates after long distance races finished by a spurt were low and by relating peak blood lactates to distances in the ski runs a smooth downward curve was obtained If the contestants in the longest race (85 km 7—8 h) were tested on a bicycle ergometer a couple of hours after finish it was found that their maximal aerobic power (max $\dot{V}_{O_2}$) was only slightly lower than before the race but even after exhaustion their peak blood lactate concentrations were only about 30 mg % (3 3 mM) as compared to 123 mg % (13 7 mM) after a test before the race Åstrand *et al* suggested a decreased glycogen store as one possible cause for the low

TABLE I

| subjects | LE | OT | PT |
|---|---|---|---|
| Age years | 22 | 25 | 24 |
| Weight kg | 56 6 | 66 7 | 67 3 |
| Height, cm | 168 | 174 | 178 |
| Max Vo l/min | 3 02 | 3 15 | 3 14 |
| Max Vo ml/min × kg | 53 4 | 47 2 | 46 7 |
| Max HR beats/min | 214 | 196 | 199 |
| signature | ○ | △ | □ |

lactate concentrations Saltin and Hermansen (1967) were able to measure the concentration of glycogen in muscle samples obtained by the needle biopsy method reintroduced by Hultman (1967) They found by varying the glycogen content experimentally that the peak blood lactates after short lasting exhaustive exercise did decrease with decreasing glycogen content but only with glycogen contents below 0 8 g/100 g muscle They found no difference in peak lactate concentrations at 0 8 g as compared to 4 g/100 g muscle However they made no actual measure ments in this range In an attempt to fill this gap Bordin Hansen (unpublished 1968) let his subjects perform short exhaustive bouts of exercise (hereafter super maximal exercise) every hour and measured the peak blood lactates after each work period The intervals were spent resting but no food was given The results from his experiments showed a steady decline in the peak blood lactates over a 12 hour experimental day some of the subjects reaching a state where exhaustive bursts of exercise did not produce any increase over the resting value of the blood lactates Karlsson (1971) after maximal exhaustive exercise preceded by long lasting exercise found that the peak blood lactate concentrations were lower than before the long lasting exercise and further that muscle lactate concentrations were lower when the submaximal exercise had lasted at least 5—7 h The muscle glycogen contents were also measured and found to have decreased to about 80 % of normal values Karlsson tentatively suggests an increased aerobic utilization of muscle and blood lactate as a possible reason for the low peak values

The purpose of the present paper was to present results from experiments in which the carbohydrate stores of the organism were experimentally varied, in order to see if the decreasing peak blood lactate concentrations after brief exhaustive exercise were correlated with the available carbohydrate and further to work out *a hypothesis that might give a causative explanation for such a correlation*

## Methods and procedure

The subjects were 3 healthy male students Individual characteristics are given in Table I Each subject went through 4 series of experiments In each series the subjects rested on a cot for about 30 min in the morning after which measurements were made of resting metabolism and blood samples w r taken for determination of lactate glucose pH plasma [K ] and [Na ] and hematocrit The subjects then performed bouts of supermaximal exercise to ex

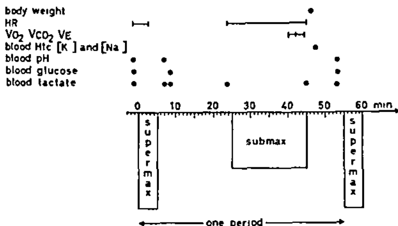



Fig 1 Schematic presentation of sampling and measuring times over one period of super maximal and submaximal exercise

haustion on a bicycle ergometer alternating with pauses of both rest and submaximal exercise (about 70 % of their maximal aerobic power) No food was given through the experimental day (On intake of glucose see below) The sequence of events is given in Fig 1 This schedule was repeated for as many hours as the subjects could sustain

The work load during supermaximal exercise was selected so as to exhaust the subjects within 2—7 min in the first bout but sometimes it was necessary later to reduce the work load somewhat in order to keep a work time of at least 2 min Also the submaximal work load occasionally had to be reduced during the experimental day

Pulmonary ventilation $O_2$-uptake and $CO_2$ output were measured by the Douglas bag method Heart rate was counted by auscultation Blood samples for determination of hematocrit (Hct) and plasma [K] and [Na] were taken from an arm vein The plasma [K] and [Na] were determined on a *Baird* flame photometer (double analysis) using plasma from the Hct determinations

Arterialized blood for determination of pH glucose and lactic acid was taken from a prewarmed finger tip Blood pH was measured by means of the *Radiometer* microelectrode type E 5021 2 or 3 determinations were made on each blood sample Double analyses of the blood glucose and lactate concentrations were carried out by means of enzymatic methods (Sholz *et al* 1959) In order to make the blood lactate concentrations following supermaximal exercises as comparable as possible 2 samples were taken at corresponding times as related to the experiment one was taken 7 min after the onset of exercise and the other one 4 min after the end of exercise The sample with the highest lactate concentration was assumed to represent the highest blood lactate concentration following supermaximal exercise ("peak lactate")

The experiments were carried out in 4 series (I to IV) on each of the 3 subjects The sequence and procedure of each series are described below under Results

## Results

*Series I* On the first day of this 2 days series the subjects worked without any intake of food or drink During the evening and night between day 1 and 2 broth tea coffee and water+salt were allowed *ad libitum* but no energy rich food was taken until after the second bout of supermaximal work on the second day when 75 g of glucose (with water) was given 30 min before a third bout of supermaximal exercise

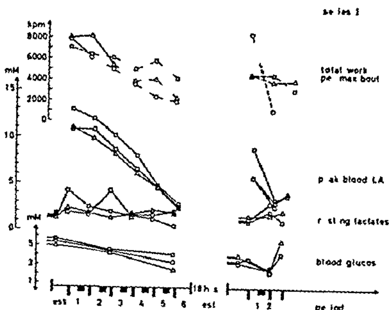



Fig. 2. Total work per supermaximal bout, blood lactate and glucose concentration on first and second day. The different signatures refer to the 3 subjects. Full lines between work outputs signify unaltered work intensity, dashed lines signify a lowering of the work intensity. After second period on second day 75 g glucose was given to two of the subjects who then performed a third bout of supermaximal exercise.

The peak blood lactate concentrations of the 3 subjects are shown in Fig. 2 with the corresponding lactate values just prior to the supermaximal tests (resting lactates, lower curves). The peak values show a steady decline from the first to the sixth period where the peak values only are slightly higher than the preceding "rest" values. On the second day the peak values after the first bout of supermaximal exercise are higher than on the previous evening, but after the second bout they are back to the same low level as at the end of the first day. The intake of 75 g glucose (2 subjects only) was followed by a rise in blood sugar and a slight increase in the peak value after a third supermaximal work.

In parallel to the declining peak blood lactates the output of work per period decreased. This took place primarily as a shortening of the time to exhaustion but later in the day the work intensity also had to be lowered in order to keep the work time longer than 2 min. The total work outputs per supermaximal period are shown in Fig. 2. They decline to about half their initial values when the increase in lactate after work has almost disappeared. One night's rest and the restoration of body weight had very little effect on the work output in subjects OT and PT and only a short lasting one in subject LE (Fig. 2).

The blood glucose was measured only at longer intervals on the first day and showed a steady decline from values between 5 and 6 mM to values close to 3 mM at the end of the first day. On the second day the blood glucose concentration was

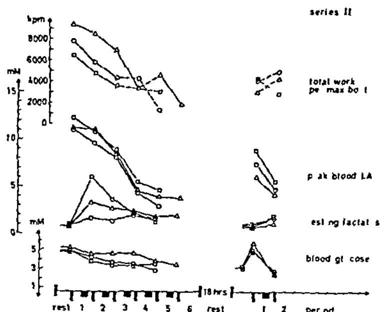



Fig 3 Total work per supermaximal bout blood lactate and glucose concentrations on first and second day Dashed lines Work intensity has been lowered On second day 10 g of glucose was given to all subjects before work period 1

slightly higher than on the previous night but two bouts of supermaximal work and the intervening period of rest and submaximal work caused a further drop in the blood glucose to about 2.5 mM Two of the subjects (OT and PT) then received 75 g of glucose orally so that the blood glucose level after 30 min of rest was back to about 5 mM before the extra third bout of supermaximal work

The body weight decreased linearly in all 3 subjects by about 2 kg during the first day but was practically restored to normal during the following evening and night Hematocrit values increased during the first day but returned to normal over night Plasma [K] and [Na] respectively decreased and increased slightly [K] was still low on the second day but [Na] was back to normal

*Series II* which was performed a couple of weeks after series I was planned exactly as series I with the exception that water was administered in the period immediately after the submaximal exercise in such amounts that the body weight was maintained In this way a general work dehydration was prevented The evening and night between first and second day of the series were as in series I i only water and electrolytes were taken *ad libitum* About 30 min before the first supermaximal test on the second day 10 g of glucose was given

The results of the series II experiments are shown in fig 3 The peak lactates after supermaximal work were almost identical with the ones in series I and showed a steady decline from about 12 mM to 3—5 mM On the morning of the second day they were somewhat higher but far from their initial values and after the

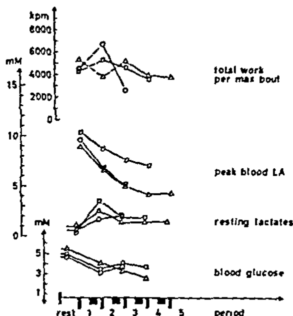



Fig 4 Same data as in Fig 3 and 4 but after 60 h on a carbohydrate poor diet

second supermaximal exercise bout they had declined again to about 5 mM The total amount of work per bout also declined in the same way as in series I Fig 3 further shows how the blood glucose concentration steadily decreased during the first day and how it was still low the next morning until 10 g of glucose ingested brought it back to normal before the first supermaximal test The last glucose values after the submaximal exercise and just before the second maximal test was however very low again The hematocrit values became slightly higher than at rest Plasma K and Na were practically constant and normal The body weight was maintained by drinking water before each supermaximal test

Series III and IV were performed in direct continuation of series II following intervals of about 60 h During these intervals the subjects were put on a special diet In the first interval—between series II and III— the subjects were given a carbohydrate low diet consisting mainly of fats and proteins In the second interval —between series III and IV— a carbohydrate rich diet was administered

The actual experiments in series III were performed as usual with the exception that besides giving water to compensate for the loss during the work periods 5 g of glucose was given orally in the morning 30 min before exercise started and further at the end of each supermaximal work test (in subjects EL and OT) or 10 min before the supermaximal test (in subject PT) This was done in an attempt to keep the blood sugar from decreasing to hypoglycemic values Furthermore there was no second day in this series

Fig 4 shows some of the results from series III It can be seen that the first peak lactate after supermaximal exercise was lower than in series I and II about 10 mM

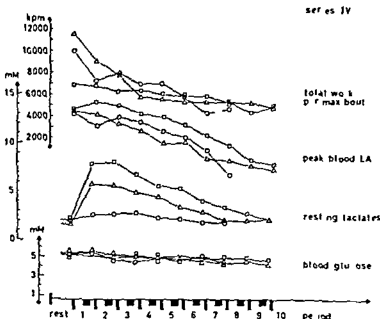



Fig 5 *Same data as in Fig 3—5 but after 60 h on a carbohydrate rich diet.*

instead of about 12 mM But the subsequent decrease followed the same curve as in series I and II with the exception of subject PT whose curve was less steep The total work before exhaustion in the supermaximal tests decreased less regularly than in series I and II, but within the same range in relation to the lactate values

The blood glucose concentration after the 60 h interval on a low carbohydrate diet was low on the morning of the experimental day but was restored to normal after the intake of 5 g glucose During the experiment it decreased as before in spite of the repeated ingestions of 5 g glucose

Despite constant body weight during series III the hematocrit showed a slight increase most markedly in subject LE (from 41 3 to 44 0 %) Plasma [K] and [Na⁺] did not change

Series IV followed directly after an interval of 60 h on a carbohydrate rich diet The procedure was the same as in series III Some results are presented in Fig 5 It is striking that the peak lactates after supermaximal exercise initially were much higher than in series III and even higher than in series I and II Further the decrease during the experimental day was much slower so that the peak lactate concentrations even after 9 h of intermittent supermaximal and submaximal exercise with a total work output of about $215 \times 10^3$ kpm as compared to about $60 \times 10^3$ kpm in series III and about $100 \times 10^3$ kpm in series II s first day were still 7—8 mM The blood glucose values started at a high resting level and decreased very little during the experimental day Body weight and hematocrit remained nearly

constant during the day Plasma [K] also was constant whereas [Na] decreased from about 145 meq/l to about 136 meq/l

Of other parameters of possible interest for the present problem the following may be mentioned The blood pH was within normal ranges in the pre exercise resting state After exercise it decreased with increasing lactate concentration but independently of the diet The heart rates—both at the supermaximal bouts and during the submaximal periods—seemed to be unaffected by the decreasing lactate release

## Discussion

The present experiments have shown in all four series on three subjects that the peak blood lactate concentration just after a bout of supermaximal exercise to exhaustion is lower the later in a series of alternating submaximal and supermaximal work periods it is measured (Fig 2—5) This finding resembles the results of Åstrand *et al* (1963) Also Bordin Hansen (1968) found that the peak blood lactates after short bouts of supermaximal exercise repeated every hour decreased gradually to resting values Even in this condition work could still be performed to amounts of 4—5000 kpm per bout at 17—1900 kpm/min Saltin and Hermansen (1967) on the other hand found that no such decrease takes place if the carbo hydrate stores of the subject are well filled but only when these are close to being emptied

Karlsson (1971) who found a decreased blood lactate concentration after maximal exercise preceded by several hours of submaximal exercise also made simultaneous determinations of muscle lactate concentrations and found that the peak values were lower than normal if the maximal test had been preceded by 5 to 7 h of heavy exercise although not after a shorter (2—4 h) preceding work period

As possible reasons for the decline in peak blood lactates after maximal or super maximal exercise several possibilities besides lowered glycogen stores may be suggested *e g* dehydration disturbances of electrolyte balance rapid oxidation of the lactate in the exercising or resting muscles and decreased enzyme activity In our series I the registered dehydration and electrolyte disturbances caused by the repeated work bouts might be considered as possible factors But the fact that the effect persisted over night in spite of adequate intake of water and salts speaks against this explanation Series II in which the loss of water was compensated and the plasma electrolytes were normal also gave exactly the same results as series I

It was observed in series I but also in series II and III that the blood glucose level was diminished during the later periods of exercise in the series—in some cases to values that gave serious subjective sensations of hypoglycemia As hypo glycemia is a well known cause of fatigue it could not be excluded from considera tion that this kind of fatigue limited the capacity for heavy exercise and thus was the direct cause of the low blood lactates Against this explanation is the fact

that Åstrand *et al* (1963) found normal glucose values after the long ski races when the peak blood lactates were very low In order to exclude hypoglycemia as a reason for fatigue in the present experiments glucose was given to the subjects In the first 2 series the blood glucose levels were raised to normal by ingestion of 75 and 10 g of glucose respectively The peak lactates after the subsequent tests were somewhat higher than after the last preceding test but still far smaller than the values at the beginning of the series In series III the small doses 5 g of glucose given in each interval apparently were too small to uphold a normal blood glucose level (Fig 4) There was however an interesting difference between the peak lactates of subject PT and those of the two other subjects which may have been caused by the glucose administration While subjects LE and OT received their glucose immediately after the supermaximal work bouts in order to facilitate the performance of the subsequent 20 min of submaximal exercise—which by all was found to be most tiresome—subject PT received his glucose 10 min before the supermaximal test and thus presumably had a rising blood glucose concentration when the test was performed His measured blood sugar concentration was not different from that of the others (*cf* Fig 4) but his peak blood lactate curve deviated from the other two subjects by showing a less rapid decline (Fig 4) Bordin Hansen (1968) in his experiments likewise found that even small amounts of sugar given late in the experimental day somewhat restored the ability to raise the blood lactate concentration It appears from this that availability of blood glucose can delay and diminish the fall in peak blood lactate concentrations

It has repeatedly been shown that the muscles content of glycogen gradually diminishes during prolonged exercise (*e g* Bergstrom and Hultman (1967)) Saltin and Hermansen (1967) pointed out that this decrease very much resembled the fall in peak lactate after maximal tests and that a causal relation between glycogen and peak lactate might therefore exist However they discarded this explanation because they failed to find a correlation between muscle glycogen and peak lactate at high values of glycogen

In the present series I and II it is justifiable to assume that the muscle glycogen stores are being gradually diminished during the experimental day The peak blood lactate concentration after exhaustion is seen to decrease in parallel to this assumed fall in muscle glycogen right from the beginning

In series III the subjects who probably had emptied their glycogen stores by exercise in series II had lived on a diet of fat and protein for about 60 h This makes it quite certain that their carbohydrate stores must have been quite low at the beginning of series III (Hultman and Bergstrom 1967) Their ability to raise the blood lactate concentration declined very rapidly (except for subject PT whose case is discussed above) In contrast—in series IV performed 60 hours after completion of series III the interval being used to fill the glycogen stores by means of carbohydrate rich food (Hultman and Bergstrom 1967)—the peak blood lactate was initially higher than usual and it declined much slower All other measured parameters—body weight electrolytes hematocrit blood sugar—remained normal

TABLE II

| diets | | Initial blood glucose mM | First max blood lactate mM* | Total work output subm +max kpm × 10 | Total work output max * kpm × 10³ |
|---|---|---|---|---|---|
| low carb hydr series III | m̄ | 4 3 | 9 7 | c 60 | c 18 |
| | range | 4 1—4 5 | 9 0—10 5 | | |
| normal series II | m̄ | 5 0 | 11 7 | c 100 | c 27 |
| | range | 4 9—5 2 | 11 3—12 4 | | |
| high carb hydr series IV | m̄ | 5 3 | 13 3 | c 215 | c 55 |
| | range | 5 2—5 3 | 13 2—13 4 | | |

* The work intensities for each subject were the same on all three diets

These facts all point strongly to the size of the glycogen stores as the determining factor for the peak blood lactate after supermaximal exercise

In parallel with the decrease in peak blood lactate the amount of supermaximal work that could be performed before exhaustion declined (Fig 2—5) This decline was due to a shortening of the work time and only in cases where this threatened to become less than about 2 min the work effect was deliberately diminished Even with the lowest peak lactate 2—5000 kpm could be performed before exhaustion defined as the state when the subject could no longer follow the metronome Also this finding resembles the results of Bordin Hansen (1968) i.e. that even in cases where the blood lactate could not be elevated over the rest value about 4000 kpm at supermaximal intensity could be performed before exhaustion If we assume that the peak blood lactate value after exercise is closely correlated to the muscle lactate (Karlsson 1971) this means that exhaustion occurred with no or a greatly reduced production of lactate

Our results have so far pointed to a close correlation between the body s content of available carbohydrates—glycogen in muscles and liver and blood glucose—and the ability to liberate energy by anaerobic glycolysis and lactate formation Some pertinent data presented in Table II demonstrate this correlation

The causes for exhaustion may be several In the present experiments exhaustion is defined as the failing ability to continue work at the prescribed rate In other words exhaustion sets in when the energy yielding processes in the muscles are no longer able to liberate energy at the preset velocity We have chosen the work power so as to make it supermaximal, i.e. higher than corresponding to the maximal aerobic power (maximal $V_{O_2}$) Some energy consequently must come from anaerobic processes among which the quantitatively most important is the breakdown of carbohydrates to lactate The velocity of this process—actually a chain of enzymatic processes—must have an upper limit, determined among other things by the concentration of 1) substrate(s) 2) enzymes and 3) the possible inhibitory effect of end products We have deliberately attempted to vary the concentration of the substrate glycogen by exercising (supermaximally and submaximally) and by diet

We have further arranged our supermaximal work periods so that the end product lactate, will accumulate in the muscles, as mirrored in the raised blood lactate concentration after the supermaximal tests

In our supermaximal tests the situation with respect to the glycolytic processes may tentatively be outlined as follows The necessary velocity of anaerobic energy liberation demands a certain concentration of the enzyme substrate complex (*Michaelis'* Theory cited from P Karlson (1968)) With high concentrations of substrate (glycogen) this can be achieved with a certain moderate amount of enzyme activated With decreasing amounts of substrate an increasing amount of active enzyme is needed to maintain the concentration of the enzyme substrate complex at the demanded level In the course of the supermaximal test lactate accumulates and produces an increasing inhibition This inhibition can be overcome over a certain time by activation of more enzyme from the stores of inactive enzyme until all enzyme finally is in the active form, whereupon the velocity of the process must decrease The time until this happens will depend on the amount of non activated enzyme *i e* it will be longer when the substrate factor in the enzyme substrate complex is large and vice versa

The above sketched development can explain our experimental findings if one assumes that the pace setting enzymatic process is the phosphorylation of glycogen to glucose 1 phosphate by means of the enzyme phosphorylase This enzyme is found in a relatively inactive b form which can be transformed into a highly active a form by a specific kinase in the presence of cyclic adenosine 3 5 mono phosphate and with ATP as P donor (P Karlson 1968)

The assumption that the limiting factor for the rapid break down of glycogen to lactate in the present experiments is the phosphorylase glycogen complex *i e* the first link in the overall process is supported by the observation that intake of gluco e enhances the ability of the muscles to produce lactate and continue work Blood glucose enters the glycogenolytic reaction chain as glucose 6 phosphate *i e* below the first step of phosphorylation Bergstrom and Hultman (1967) also found that infusion of glucose during muscular exercise resulted in a slower deple tion of the muscle glycogen stores

The problem of the decreasing ability to liberate energy by glycogenolysis and lactate production after prolonged exercise after repeated bouts of supermaximal exercise and after a period on a carbohydrate poor diet accordingly can be ex plained as the results of an inhibition on the glycogen—glucose 1 phosphate reac tion caused by accumulation of lactate The inhibitory effect of a given lactate concentration will be relatively stronger the lower the concentration of the substrate glycogen is

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# Changes in the Concentration and Fatty Acid Composition of Phospholipids in Rat Skeletal Muscle during Postnatal Development

By

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## Abstract

Bruce A *Changes in the concentration and fatty acid composition of phospholipids in rat skeletal muscle during postnatal development* Acta physiol scand 1974 90 743—749

Rat skeletal muscle was analysed at different ages from birth to 90 days for its concentration of phospholipids and individual phosphoglycerides and for the fatty acid pattern of cardiolipin choline phosphoglyceride (CPG) ethanolamine phosphoglyceride (EPG) inositol phosphoglyceride and serine phosphoglyceride (SPG) The concentration of phospholipids was found to increase between birth and two weeks of age after which it decreased slightly to 14 μmol/g fresh weight at the age of 90 days The relative amount of CPG and EPG varied but little with age while that of cardiolipin increased to three times its original value The concentration of polyenoic acids increased with age in all phosphoglycerides except SPG The concentration of the sum of the fatty acids of the linoleic acid series in CPG increased rapidly during the first weeks of life while that of the linolenic acid series increased throughout the period studied In EPG the concentration of the sum of the fatty acids of the linolenic acid series increased while that of the linoleic acids decreased and the concentration of linoleic acid in cardiolipin increased In the muscles of 90-day-old rats the fatty acid pattern of cardiolipin showed a high level of linoleic acid and no fatty acids of the linolenic acid series The fatty acid pattern of CPG was characterized by rather high levels of both linoleic and arachidonic acids and that of EPG by a high concentration of the fatty acids of the linolenic acid series

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Studies on the phospholipid composition of the human skeletal muscles have revealed characteristic age variations in the composition of phospholipids and the fatty acid pattern of the individual phosphoglycerides (Bruce and Svennerholm 1971 Bruce 1974 a b) The most pertinent finding was a large increase in the concentration of essential fatty acids during gestation and early postnatal period mainly in choline phosphoglyceride but also in cardiolipin and ethanolamine phosphoglyceride

*Abbreviation* Fatty acids are designated by chain length number of double bonds *n*-6 denotes that the first double bond from the methyl group occurs after the sixth carbon atom the methyl group being counted as number 1 CPG choline phosphoglyceride EPG ethanolamine phosphoglyceride IPG inositol phosphoglyceride SPG serine phosphoglyceride

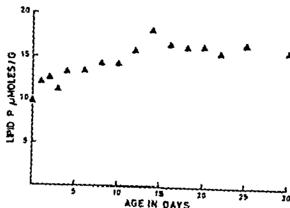



Fig 1 Concentration of total phospholipids in rat skeletal muscles expressed in µmol/g wet wt

Comparable data from humans and other mammals were very scarce It was therefore difficult to ascertain to what extent these findings represented a general biological pattern and to what degree they were the result of nutritional influences particularly in the supply of essential fatty acids In order to elucidate these questions rats were studied This paper reports the age variation of the phospholipids in rats fed an ordinary laboratory diet As only scattered data are available on the phospholipid composition of the rat muscles (Froberg 1967 Kanoh 1967 Borchot and DiCostanzo 1968 Patriarca *et al* 1969 Simon and Rouser 1969) the study was extended to include a rather detailed survey of the phospholipids their fatty acid patterns and their variation with age from birth to 90 days

## Materials and methods

*Animals* Sprague Dawley rats fed a standard pelleted diet (Anticimex 210 Evelund S 191 71 Sollentuna Sweden) were used The caloric composition of the diet was protein 21 % fat 10 % and carbohydrates 69 % Linoleic and linolenic acids constituted approximately 3 % of the calories and the ratio between linoleic and linolenic acid was 4 1 The litters were reduced to six animals on the day of birth At 0 4 and 10 days of age individuals from one litter were killed at the age of 18 days 3 males and 3 females and at 30 45 and 90 days one male and one female In order to elucidate in detail some of the variations found in the lipid composition during the first 30 days of life muscles from one newborn litter and from litters aged 1 2 3 4 6 8 10 12 and 14 days were obtained and analysed At the ages of 16 18 20 22 25 and 30 days an average number of 4 animals of each age were studied

The rats were killed by decapitation under ether anesthesia and bled Samples of the quadriceps and gastrocnemius muscles were excised and analyzed at once or stored at −20 C until analysis The muscle samples from rats aged 18 30 45 and 90 days in the first series were grouped according to sex otherwise all muscles from rats of the same age were pooled From litters up to 6 days of age 0 1—0 2 g of muscle tissue from each litter was analysed from rats between 8—18 days of age about 0 5 g and from rats older than 20 days of age about 1 g

*Analytical* The lipid extraction isolation of individual phosphoglycerides and determination of their fatty acid patterns have been described (Bruce and Svennerholm 1971 Bruce 1974 b) Phosphorus was determined according to Svennerholm and Vanier (1972) and cholesterol according to Crawford (1958)

TABLE I Phospholipid composition and fatty acid pattern of individual phosphoglycerides r 90-day-old rats

| | Cardiolipin | CPG | EPG | IPG | SPG |
|---|---|---|---|---|---|
| Phospholipid composition[a] ( of total lipid P) | 10 | 52 | 22 | 7 | 5 |
| Fatty acid | Mole % of total fatty acids | | | | |
| 16 0 | 1 | 38 | 7 | 3 | 8 |
| 16 1 | 1 | 1 | —[b] | — | — |
| 18 0 | 1 | 7 | 28 | 47 | 43 |
| 18 1 | 7 | 8 | 4 | 2 | 5 |
| 18 2 (n−6) | 89 | 20 | 7 | 2 | 4 |
| 20 3 (n−6) | 1 | 1 | — | 3 | 2 |
| 20 4 (n−6) | 1 | 13 | 9 | 29 | 9 |
| 22 5 (n−3) | — | 1 | 3 | 3 | 3 |
| 22 6 (n−3) | — | 10 | 41 | 9 | 25 |
| 18−22 (n−6) | 90 | 35 | 18 | 35 | 17 |
| 18−22 (n−3) | — | 11 | 44 | 12 | 28 |

[a] Sphingomyelin constituted 4 of total phospholipids
[b] — indicates that the actual fatty acid constituted less than 0 5 of the total fatty acids

## Results

### *Phospholipids and cholesterol*

The concentrations of lipid phosphorus up to the age of 30 days are given in Fig 1 After this age no major changes were found When the phospholipids were related to lipid and water free residue about 120 μmol per g residue was found at birth The values then decreased to 70 μmol per g residue at the age of 90 days There was an early increase in the concentration of cholesterol from 4 μmol per g fresh weight at birth to 7 μmol at the age of 10 days After this age there was a continuous decrease to approximately 2 5 μmol per g fresh weight at the age of 90 days Neither the concentrations of phospholipids nor that of cholesterol showed any difference with sex.

### *Quantitative determination of individual phospholipids*

The phospholipid composition of the skeletal muscles from one litter each of new born 4 10 and 18 day old rats and from males aged 30 45 and 90 days was determined. The composition in the 90 day-old rat is given in Table I The proportion of the major phosphoglyceride CPG was very constant with age and constituted between 48 and 52 % of the total phospholipids EPG was also constant the range of variation being between 22 and 28 % The relative amount of IPG varied between 6 and 8 % but the average level remained constant The SPG increased slightly from 5 % at birth to 7 % at 10 days of age after which it fell to 4 % at 90 days of age

For cardiolipin and sphingomyelin slight but clear changes with age were found There was an increase in cardiolipin from 4 % of total lipid phosphorus in the

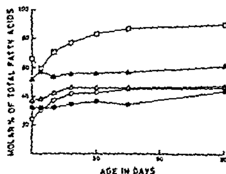



Fig 2

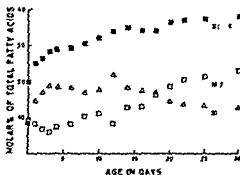



Fig 3

Fig 2 Concentration of the sum of polyenoic acids in cardiolipin (□) CPG (O) EPG (▲) IPG (△) and SPG (●) of rat skeletal muscle at different ages

Fig 3 Concentration of the sum of the fatty acids of the linoleic acid series (■) linoleic acid (□) and arachidonic acid (△) in CPG of rat skeletal muscle

born rat to 10 % at the age of 90 days while the proportion of sphingomyelin decreased continuously from 8 % in the newborn to 4 % at 90 days of age

*Fatty acid composition of individual phosphoglycerides*

The fatty acid compositions of cardiolipin CPG and EPG were determined separately for males and females at 18 30 45 and 90 days of age but no characteristic differences were found between the sexes In newborn 4 and 10-day-old rats males and females were analysed together as were IPG and SPG at all ages The fatty acid composit on of the individual phosphoglycerides of 90-day-old male and female rats are given in Table I Fig 2 gives the sum of the fatty acids of the linoleic and linolenic acid series from the phosphoglycerides at different ages

*Choline phosphoglyceride* The percentage of linoleic and arachidonic acid and that of the sum of the fatty acids of the linoleic acid series up to 30 days of age are given in Fig 3 After this age and up to 90 days of age only minor variations were found

The concentration of the polyenoic acids increased This was due mostly to a rather rapid increase in the sum of the linoleic acids during the first 3 weeks of life There was also a slow increase in the sum of the fatty acids of the linolenic acid series but this was a continuous process the highest value being found at 90 days of age The concentration of arachidonic acid was higher than that of linoleic acid during the first 18 days of life On the 3rd postnatal day the concentration of arachidonic acid was twice that at birth after which it slowly decreased to the age of 30 days After the age of 18 days linoleic acid was the dominating polyenoic acid The other fatty acids of the linoleic acid series 20 3 and 22 4 never exceeded 2 5 % The variation in the concentration of the linolenic acid series was due to a continuous rise in the level of 22 6 from birth when it was 3 % After the age of 1 day 22 5 (n—3) constituted 1 % and showed a slight increase with a maximum value of 2 5 % at 18 days of age

The sum of the saturated fatty acids remain almost constant during the whole period studied while oleic acid decreased during the first 18 days of life Appreciable amounts of 20 3(n—9) were found during the first postnatal days but this fatty acid diminished rapidly from 1 5 % at birth to 0 2 % at 12 days of age

*Cardiolipin* The fatty acid pattern of this phosphoglyceride was characterized by high levels of linoleic acid while fatty acids of the linolenic acid eries were absent At birth 20 3(n—6) and 20 4(n—6) both constituted 6 % but both fatty acids diminished at higher ages Linoleic acid increased from 55 % at birth to 85 % at the age of 45 days The increase in the concentration of linoleic acid was accompanied by a reduction in the concentrations of palmitic stearic and oleic acid

*Ethanolamine phosphoglyceride* While only a slight increase with age was found in the total concentration of the polyenoic acids there was a pronounced increase in th sum of fatty acid of the linolenic acid series from 22 % to 44 % during the period studied Meanwhile a decrease was found in the concentration of the sum of fatty acids of the linoleic acid series from 31 % to 18 % The variations in the concentration of linoleic and arachidonic acid were similar to those in CPG with a rapid increase in the concentration of arachidonic acid during the first 4 days of life followed by a continuous decrease The increase in the sum of the fatty acids of the linolenic acid series was due to a continuous increase in the percentage of 22 6 while 22 5 increased only from birth to 18 days of age Oleic and palmitic acid decreased continuously, while stearic acid increased

*Inositol phosphoglyceride* During the first 18 days there was an increase in the concentration of polyenoic acids This was due to an increase in the linoleic as well as in the linolenic acid series After this age no variations was found During the first 10 days of life there was a slight increase in the concentration of arachidonic acid while linoleic acid remained almost constant A continuous reciprocal variation in the concentration of palmitic and stearic acid was found palmitic acid decreasing and stearic increasing Oleic acid decreased continuously from birth and about 1 % of 20 3(n—9) was present at all ages

*Serine phosphoglyceride* The concentration of polyenoic fatty acids was constant During the first ten days there was a slight increase in the sum of the linoleic fatty acids while that of the linolenic acids decreased The dominating fatty acid of the linoleic acid series was arachidonic acid while 22 6(n—3) dominated the linolenic acid series

## Discussion

Newborn rats are more immature than newborn humans In the brain it is possible to distinguish certain developmental periods common to man and the rat (McIlwain 1966) During the second of these periods the brain undergoes a growth spurt in rat this period occurs during the first 10 postnatal days while in humans it takes place between the 25th fetal week and term Histological studies on human and rat muscles have revealed that in some respects this timing in the development of th brain has a counterpart in the development of muscle tissue In man the format of new muscle fibres seems to cease within 4 months after birth Montgomery

and in rat within about 3 weeks (Chiakulas and Pauly 1965). In man the differentiation of the muscle cells into red and white fibres ends at term and in rat at the age of 7—10 days (Dubowitz 1963).

These findings indicate that, from a morphological point of view, it might be justified to compare muscles from newborn rats with those from approximately 25 week old human fetuses and from 10 day-old rats with those from newborn humans. Before the birth of the rat and the 25th gestational week of the human fetus the individuals of both species have been nourished via the placenta. The human fetus then continues to be nourished *in utero* while the newborn rat suckles milk. The milk has a high fat content and provides the young rat with fatty acids most probably differing in amount and composition from those supplied to the human fetus (Alling *et al.* 1974 a).

The concentration of phospholipids in skeletal muscle was almost the same in adult humans and 90 day-old rats whether expressed in $\mu$mol per gram fresh weight (12 and 14 respectively) or in $\mu$mol per gram lipid- and water-free residue (60 and 70 respectively) (Bruce and Svennerholm 1971). A similar variation with age in the concentration of phospolipids was found in man and in the rat: a moderate increase up to a certain age (1 year in man and 2 weeks in the rat) followed by a slow decrease. When the phospholipids were related to lipid- and water-free residue the values at the lowest ages studied in both species were about 120 $\mu$mol per g. With increasing age these values showed an initial rapid decrease which gradually ceased.

There was no variation with age in the percentage of CPG and EPG of total phospholipids in either species (Bruce 1974 a). Cardiolipin increased, the highest values being found at the age of 1 year in humans and 90 days in the rats. The proportions of SPG and sphingomyelin decreased in both species. In adult humans and rats the relative amounts of the individual phospholipids were essentially the same and compatible with the values given by Simon and Rouser (1969).

Several similarities were found in the fatty acid pattern of the individual phosphoglycerides in adult muscles of both species. The total concentration of polyenoic acids was of the same magnitude in both species. A general feature was that the concentration of the linolenic acid series was higher in the rats than in the humans. This was particularely evident in EPG and SPG which in humans contained more fatty acids of the linoleic than of the linolenic acid series while the opposite was found in the rat. A possible explanation for these findings was differences in the levels of linolenic acid between the human and the rat diets. The Swedish diet contained 0.3—0.4 cal % of linolenic acid (Eeg Larsen 1966) compared to 0.6 cal % in the rat diet. The differences in ratio between linoleic and linolenic acid might also have influenced the differences in the fatty acid patterns. In the rat diet the ratio was 4 : 1 while in the human diet it was more than 6 : 1.

In both species the development changes in the fatty acid pattern of the individual phosphoglycerides were most evident in cardiolipin, CPG and EPG. The concentration of the linoleic acid series in both cardiolipin and CPG increased with age in humans as well as rats. In EPG the differences between the levels of linoleic and

linolenic acid in the diets also influenced the variation with age in man the concentration of the linoleic acid series increased during the gestational period while barely any increase was noted in the linolenic acid series In the rat the linoleic acid series increased only during the first days of postnatal life while the linolenic acid series steadily increased throughout the period studied When 24—30 week old human fetuses were compared with newborn rats the fatty acid pattern of CPG in muscles from both species were similar A characteristic feature of the early age variation in the fatty acid pattern of CPG was the increase in the concentration of arachidonic acid In the humans this fatty acid reached maximum values shortly before birth and in the rats at 4 days of age The similarity in time of this striking increase suggested the possibility that the increase was related to some stage of morphological development.

The major features of the age variations of the phosphoglycerides were similar in humans and rats when consideration was taken to different timings of the muscle development The differences found were mainly in the concentrations of fatty acids of the linolenic acid series and these differences were probably attributable to differences in the diets Whether and to what degree the fatty acid pattern of lecithin in rat muscles is influenced by the supply of linoleic and linolenic acid in the diet will be the subject of a forthcoming paper (Alling *et al* 1974 b)

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# The Influence of Temperature on the Force-Velocity Relationship in Rabbit Papillary Muscle

By

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## Abstract

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The force-velocity relationship was determined at the *moment of isometric peak twitch force* in rabbit papillary muscles using the damped-release technique described previously. The analysis was carried out in each muscle at two or three temperatures within the range 22—33° C. The experimental data were fitted with Hill's equation by means of a computer program using the least squares method. Lowering of the temperature caused an increase of the isometric peak twitch force ($P_0$) and also generally an increase of the extrapolated value of $V_{max}$ (the shortening velocity at zero load). The temperature-induced changes of the force-velocity curve were interpreted as being the net result of two opposing effects 1) an altered degree of activation of the contractile system caused by a change in the amount of activator calcium bound to the contractile proteins (*inotropic temperature effect*) and 2) a change in the rate of interaction between the A and I filaments independent of the degree of activation (*specific temperature effect*). The analysis indicated a $Q_{10}$ of 1.9 for the specific temperature effect. This value accords with the temperature dependence of $V_{max}$ that has been observed during tetanic contraction of skeletal muscle

It is well known that temperaure reduction within the range 20—37° C increases the isometric force of myocardial preparations that are stimulated to contract at a low frequency (Trautwein and Dudel 1954 Kaufmann and Fleckenstein 1965 Šumbera Kruta and Braveny 1966 Langer and Brady 1968). This temperature induced inotropic effect has been ascribed to an alteration in excitation contraction coupling involving an increase in the amount of activator calcium that is released into the myoplasm in response to the action potential (Kaufmann and Fleckenstein 1965 Langer and Brady 1968)

For a more complete understanding of the temperature effects on the contractile events it was of interest to determine the influence of temperature on the force

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velocity relationship in cardiac muscle To this end the force velocity curve has been analyzed at the instant of isometric peak twitch tension in rabbit papillary muscles at temperatures varied between 22° C and 33° C Evidence will be presented to show that the temperature induced changes of the force velocity curve involve two distinct effects on the contractile process an altered degree of activation of the contractile system and a specific change of the relationship between force and velocity of shortening of the active unit A brief account of these results has been presented previously (Edman Mattiazzi and Nilsson 1973)

## Methods

*Preparation and mounting* Papillary muscles from the right ventricle of heparinized rabbits (weight < 1 2 kg) were used The techniques used for dissection and mounting of the muscles have been described (Edman and Nilsson 1968) The muscle was mounted vertically in a jacketed Perspex chamber The base of the muscle together with a piece of the ventricular wall was attached to a tension transducer (RCA 5734) which was fitted in the chamber The upper (tendinous) end of the muscle was connected via a straightened steel wire to an isotonic lever A preload of 1 0—1 5 mN was used The preloaded length *i e* the distance between the insertions of the muscle to the tendon and the ventricular wall and the diameter of the muscle were determined with a microscope at 10× and 40× magnification respectively with the muscle in the bath The muscles had an approximately cylindrical shape with the upper 10—15 per cent of the length (next to the insertion to the tendon) being tapered The length of the preparations was 5 5±0 2 mm and their diameter at the base was 0 7±0 1 mm both values representing the mean±standard error of the mean

The temperature of the bath was kept constant to ±0 3 C throughout a given experiment by circulating water from a thermostated tank through the jacket of the muscle chamber

*Recording arrangement* A detailed description of the recording device and the techniques used for stimulation of the muscle production of release and recording of the signals from the transducers has been given previously (Edman and Nilsson 1968 also see Edman and Nilsson 1972) Loading of the isotonic lever was achieved by means of a coil spring attached to a point close to the fulcrum (lever ratio 1 .0) Two different levers (I and II) of similar design were used during this study The equivalent mass of levers I and II (including the steel wire connection between muscle and lever and the coil spring used for loading of the lever) was 91 mg and 156 mg respectively The static friction of the levers was < 0 02 mN The compliance of the lever tension transducer and all connections to the muscle was < 2 μm/mN

*Damping of oscillations* A dash pot placed on the same side of the fulcrum as the muscle was used to damp oscillations of the lever (see Edman and Nilsson 1972) Using a silicon fluid of appropriate viscosity in the dash pot (6000 centistokes) it was possible to limit the release induced oscillation in shortening velocity to ±0 1 lengths/s which corresponds to approximately ±5 per cent of the steady shortening velocity recorded at a light load As was demonstrated previously (Edman and Nilsson 1972) this degree of damping prevents the muscle from being deactivated during the release procedure This arrangement is called the *damped release technique* in order to distinguish it from conventional (undamped) quick release approaches

*Frequency response of the isotonic lever* When the system was properly damped (see above) the equivalent mass of levers I and II was increased to 180 mg and 250 mg and their natural frequency calculated to be 265 Hz and 233 Hz respectively (Edman and Nilsson 1972) A further diminution of the resonant frequency occurred after mounting the muscle The frequency of oscillation after release of the muscle when the levers were slightly under damped was in the range 60—80 Hz

*Stimulation frequency and temperature* The muscle was stimulated to contract isometrically at a frequency of 10 to 20 per min for approximately 1/2 h and thereafter at a frequency of 30 per min for at least 1 h before the experiment was started All results refer to steady state conditions with respect to frequency and temperature *i e* they were obtained after the muscle had been paced at a constant rate for at least 15 min at the temperature studied The stimulation frequency used during the experiments is given under Results

*Analysis of experimental data* The force velocity data were fitted with Hill s equation

(1938) by means of the least squares method using a computer program (Edman and Nilsson 1972) The Hill equation (1938) is given by

$$(P+a)\ (V+b) = b(P_0+a)$$

in which P denotes force V velocity of shortening and $a$ and $b$ are constants with dimensions of force and velocity respectively $P_0$ is the force at zero velocity of shortening

Student s t test was used in the statistical analysis Measurements from film records were carried out in a Nikon comparator at 10 or 20× magnification

*Solutions* A Ringer solution of the following composition was employed (mM) NaCl 117 0 KCl 4 0 $NaHCO_3$ 20 0 $NaH_2PO_4$ 1 5 $CaCl_2$ 2 0 $MgSO_4$ 1 4 glucose 3 3 The solution was continuously aerated with a mixture of 95 % $O_2$ and 5 % $CO_2$ during dissection of the muscle as well as during the actual experiment The pH of the solution was 7 4—7 5

Glass distilled water was used for washing of glassware and for preparation of solutions All chemicals used were of analytical grade

## Results

In the following description of the force velocity relationship the notations given in Hill s equation (1938) are used (see Methods)

Force velocity curves were obtained by releasing papillary muscles to various loads just prior to the peak of the isometric twitch and recording the shortening velocities as described in detail previously (Edman and Nilsson 1968 1972) All velocity data for each curve were obtained at the precise moment of the isometric peak twitch tension Under these conditions the isometric peak twitch tension provided an appropriate value for $P_0$ (the force at zero velocity of shortening) as it referred to the same instant during the activity period as the velocity data obtained by releasing to various loads

6 expts were performed at a constant contraction frequency (which varied between 30 and 48 per min in the different experiments) at 2 or 3 temperatures within the range 22—33° C Higher frequencies generally prevented the muscle from relaxing completely at temperatures lower than 26° C Another 4 expts were carried out between 22° C and 33° C but in these experiments the contraction frequency was altered from 45—54 per min at the higher temperature to 18—24 per min at the lower temperature The object of changing the frequency was to minimize the difference in $P_0$ between the two temperatures As previously demonstrated (Edman and Nilsson 1969 Mattiazzi and Nilsson 1973) a change in contraction frequency *per se* does not affect the constants $a$ and $b$ in Hill s equation These latter experiments thus provide useful information for determination of the effect of temperature on the shape of the force velocity curve

Fig 1 A and 2 A illustrate the results of 2 expts at constant contraction frequency It can be seen that lowering of the temperature from 32° C to 23° C increased the value of $P_0$ two to four times The extrapolated value of $V_{max}$ was also increased However the fractional increase in $P_0$ was greater than that of $V_{max}$ In some cases $V_{max}$ was found to decrease slightly as the temperature was reduced (cf $t_2$ and $t_3$ in Fig 2 A)

*Analysis of the temperature effects* The temperature induced change in the force velocity curve is fundamentally different from that produced by inotropic interventions such as changes in contraction frequency (Edman and Nilsson 1969 Mattiazzi and Nilsson 1973) increased calcium concentration or addition of cardiotonic

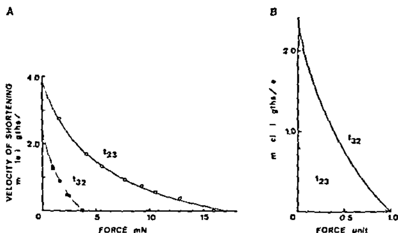



Fig 1 A Force velocity curves of a rabbit papillary muscle obtained by means of damped release method at 32.0 C ($t_{32}$) and 22.8 C ($t_{23}$) Curves fitted by means of Hill's equation (1938) using the following values for the constants $a$ and $b$ $t_{32}$ $a = 3.4$ mN $b = 2.11$ muscle lengths per s $t_{23}$ $a = 5.9$ mN $b = 1.31$ muscle lengths per s Contraction frequency 30 per min Muscle length 4.0 mm. Muscle diameter 0.4 mm B Force velocity curves shown in A after correction for temperature induced change in the degree of activation of the contractile system Curve $t_{32}$ (control) is identical with the corresponding curve in A Curve $t_{23}$ has been constructed by using the $P_0$ value of curve $t_{32}$ and the constants $a$ and $b$ of curve $t_{23}$ in A For further information see text

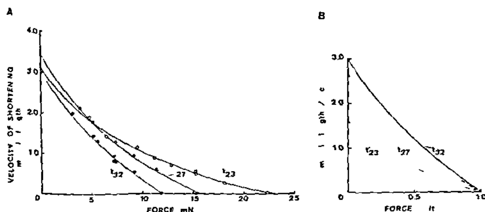



Fig 2 A Same type of experiment as in Fig 1 Curves $t_{32}$ $t_{27}$ and $t_{23}$ obtained by means of damped release technique in a rabbit papillary muscle at 32.0 C 27.5 C and 22.8 C respectively The constants $a$ and $b$ of Hill's equation (1938) used for construction of the curves were $t_{32}$ $a = 23.3$ mN $b = 5.31$ muscle lengths per s $t_{27}$ $a = 17.7$ mN $b = 3.6$ muscle lengths per s $t_{23}$ $a = 14.8$ mN $b = 1.99$ muscle lengths per s Contraction frequency 36 per min Muscle length 4.5 mm Muscle diameter 0.7 mm B Force velocity curves shown in A after correction for temperature induced change in the degree of activation of the contractile system Curve $t_{32}$ (control) is identical with the corresponding curve in A Curves $t_{27}$ and $t_{23}$ have been constructed using the $P_0$ value of curve $t_{32}$ and the constants $a$ and $b$ of curves $t_{27}$ and $t_{23}$ shown in A

drugs (Mattiazzi and Nilsson 1973). In all these cases $V_{max}$ and $P_0$ are affected to the same degree with no apparent change in the constants $a$ and $b$ in Hill's equation.

We consider the temperature induced changes in the force-velocity curve to be the net result of two effects. For the purposes of the present analysis it is assumed that temperature affects 1. the degree of activation of the contractile system, *i.e.* the amount of calcium bound to the contractile proteins during the contraction cycle (*inotropic temperature effect*) and 2. the rate of interaction between the A and I filaments (*specific temperature effect*) independent of the degree of activation.

The inotropic temperature effect is expected to take the form of a change in $P_0$ with no change in the constants $a$ and $b$ of Hill's equation. Hence the dashed $t_{-3}$ and $t_{-7}$ curves in Fig. 1 B and 2 B have been constructed by shifting the $P_0$ values of curves $t_{-3}$ and $t_{-7}$ to coincide with the $P_0$ of curve $t_3$. The same respective values of $a$ and $b$ have been utilized for construction of curves $t_{-3}$ and $t_{-7}$ as used to fit the hyperbolae to the $t_{-3}$ and $t_{-7}$ data in Fig. 1 A and 2 A. By this procedure the inotropic temperature effect, *i.e.* the changes in $P_0$ and $V_{max}$ resulting from altered degree of activation, have been removed from the curves in Fig. 1 B and 2 B. The difference in $V_{max}$ existing after this normalization of the force-velocity data provides an index of the specific temperature effect.

It is seen from Fig. 1 B and 2 B that decreasing the temperature produces a substantial decrease in the normalized value of $V_{max}$ which, when analyzed in the different experiments, indicates a $Q_{10}$ of 1.9 ± 0.3 (mean ± S.E., $n = 10$).[1]

[1] In this analysis it is implicitly assumed that $P_0$ at a given degree of activation of the myocardium is independent of temperature. Available data seem to suggest that in mammalian skeletal muscles $P_0$ (measured as maximum isometric tetanic tension) has a $Q_{10}$ of 1.0–1.2 within the temperature range 20–37° C (Truong *et al.* 1964, Close 1965, Close and Hoh 1968, Gabel *et al.* 1968). If a $Q_{10}$ of 1.2 were also to apply for $P_0$ in myocardium, the $Q_{10}$ of $V_{max}$ estimated above would be 1.2 times greater than the figure obtained, *i.e.* 2.3.

## Discussion

In the present study the force-velocity curve has been determined at a constant length of the contractile element by means of a release technique that has been shown previously to give an insignificant amount of distortion of the curve (Edman and Nilsson 1972). The positive inotropic change produced by temperature reduction affected $V_m$ to a smaller degree and in some cases even in the opposite direction than $P_0$. This is in contrast to other inotropic interventions (brought about by adrenaline, ouabain, increased extracellular calcium concentration and increased contraction frequency) where parallel changes in $V_{max}$ and $P_0$ occur without significant alterations in the shape of the force-velocity curve as judged by the constant numerical values of $a$ and $b$ of Hill's equation. Our analysis has shown that the temperature induced alteration of the force-velocity curve is probably the net result of two different effects. 1. An inotropic temperature effect of the same nature as that produced by other inotropic interventions and which in itself affects $P_0$ and

$V_{max}$ to the same extent without altering the shape of the force velocity curve The inotropic effect of temperature can be ascribed to an increase in the *concentration* of activator calcium at the contractile sites (*cf* discussion in Edman and Nilsson 1969) 2) *After allowing for the inotropic change under point 1 a specific temperature effect* can be demonstrated By *this effect* $V_{max}$ is altered relative to $P_0$ independent of the degree of activation According to this analysis $V_{max}$ increases with *temperature* with a $Q_{10}$ of 1 9 (or 2 3 see *further page 754*)

This specific *temperature effect* agrees quantitatively with the temperature dependence of $V_{max}$ recorded in skeletal *muscle during tetanic contraction* $Q_{10}$ values of *approximately 1 7 have* been obtained for $V_{max}$ in various mammalian skeletal *muscles* within the temperature range 25—35° C (*Close 1965*) whereas values of 2 0 (Hill 1938) and 2 6 (Julian Steeber and Sollins 1971) have been recorded in *amphibian* skeletal muscle Considerably lower $Q_{10}$ values *have been measured* for $P_0$ (between unity and 1 2) *in both mammalian* (Truong *et al* 1964 Close 1965 Close *and Hoh 1968* Gabel *et al* 1968) and amphibian (Hill 1951 *Close and Hoh* 1968) skeletal muscle It should be *noted that the* $Q_{10}$ values of $V_{max}$ in skeletal *muscle refer to a constant state of activity* of the contractile system (*tetanic stimulation*) They are therefore comparable *with the* $Q_{10}$*-value of the* specific temperature effect on $V_{max}$ recorded in the present analysis where allowance *has been made for* the inotropic effect The results would thus *seem to indicate that* $V_{max}$ evaluated at a constant *degree of activation of the* contractile system has a similar temperature dependence in skeletal muscle and myocardium

$V_{max}$ the shortening velocity at zero load is *likely to reflect the maximum* rate of interaction between *the A and I filaments Evidence* in favour of this idea is provided by the observation that $V_{max}$ is quantitatively related to the myosin ATPase activity (determined *in vitro*) *in skeltal muscle of* a variety of mammals *lower vertebrates* and invertebrates (Barany 1967) It is of further interest to note *in this* connection that Barany no the basis of his *in vitro* studies estimated the $Q_{10}$ of actomyosin *ATPase activity in frog* skeletal muscle to be 2 0 This figure is close to the *measured* $Q_{10}$ value of $V_{max}$ (*cf* above) in amphibian skeletal muscle

The temperature dependence of the *force* velocity curve in myocardium of frog (Mashima *and* Matsumura *1964*) and cat (Yeatman Parmley and Sonnenblick 1969) *has* been analyzed previously using afterload *recording techniques In both* these studies contrary *to* the findings reported above (Fig 1 A and 2 A) the measured values of $V_{max}$ were found to increase considerably with temperature A quantitative comparison between our own *results and those obtained* previously is not feasible, however The force velocity curve derived by means of afterload recording *technique* is based on measurements which refer to different times during the contraction cycle and hence to difference *degrees of activation* of the contractile unit The temperature induced change in $V_{max}$ of this curve therefore reflects not only the inotropic and specific temperature effects described here *but also the* alteration in the time course of the *mechanical activity* (*Trautwein* and Dudel 1954 Kaufmann and *Fleckenstein 1965* Langer and Brady 1968)

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# Insulin Sensitivity in Rats with Ventromedial Hypothalamic Lesions

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**Abstract**

FAHLE HONGSLO C B E HUSTVEDT and A. LOVO *Insulin sensitivity in rats with ventromedial hypothalamic lesions* Acta physiol scand 1974 90 757—763

The insulin sensitivity of the diaphragm and parametrial adipose tissue in animals with ventromedial hypothalamic (VMH) lesions and controls were assayed *in vivo* by studying the intraperitoneal action of exogenous insulin Lipogenesis and glycogen synthesis were stimulated to the same extent in lesioned and control animals This implies that the co-existence of hyperinsulinemia and normoglycemia previously demonstrated in lesioned animals cannot be attributed to decreased insulin sensitivity Increased gluconeogenesis in the lesioned animals is indicated by elevated levels of plasma urea. This may contribute to the maintainance of normoglycemia in the presence of hyperinsulinemia and normal insulin sensitivity It is suggested that the profound metabolic effects of VMH destruction is caused by a disturbance in the nervous control of gastro-intestinal functions This may lead to alterations in the endocrine pancreatic secretion and possibly also in liver metabolism

Ventromedial hypothalamic lesions (VMH) in the adult rat give rise to hyperphagia and extensive accumulation of body fat (Hetherington and Ranson 1939) Animals with such lesions exhibit increased plasma insulin levels (Hales and Kennedy 1964) Hyperinsulinemia is present already 2 days post-operatively even when hyperphagia is prevented (Hustvedt and Lovo 1972) The described increase in plasma insulin concentration is therefore not the result of adaptive metabolic changes secondary to hyperphagia

The presence of hyperinsulinemia in human obesity as well as in genetically obese laboratory animals is well known and has been regarded as secondary to a resistance by peripheral tissues to the insulin action The question has therefore been raised as to wether the VMH lesions lead to nervous and/or endocrine disturbances that may result in increased insulin resistance in some tissue(s) However recent publications dealing with these problems report responses to insulin in the normal range in diaphragm as well as in adipose tissue (Frohman *et al* 1972 Han *et al* 1972) Frohman *et al* (1972) studied the action of endogenous and exogenous insulin

the VMH lesioned weanling rat 3 weeks after surgery, while the work of Han *et al* (1972) was performed on 200 g hypophysectomized rats 21 days post-operatively

It is known however that the metabolism of the VMH lesioned animal is changed already a few hours post-operatively so as to promote accumulation of body fat at the expense of other anabolic processes (Hustvedt and Løvø 1973 Holm *et al* in press) These animals will during the 3 first post-operative weeks accumulate significantly more lipids and less protein and water than their controls even on a normalized food intake (Holm *et al* in press) The magnitude of this increased lipid accumulation is in the order of 100 % The obesity caused by VMH destruction is characterized by marked enlargement of the fat cells with little or no change in cell number (Hirsch and Han 1969) This enlargement will probably alter the insulin sensitivity of the fat cells because it is evidence for an inverse correlation between cell size and response to insulin *in vitro* by adipose tissue (Salans *et al* 1968)

In order to evaluate the effect of VMH destruction *per se* on insulin sensitivity it therefore seems important to carry out such studies as early after the operation as possible The present expriments were performed 2 days post-operatively on animal kept on a normalized food regimen after surgery

## Materials and methods

*Animals and animal care*

Female Wistar rats AF/Han/MØ/Han) weighing 160—180 g were used The animals were kept in separate cages during the experimental period The lighting of the animal room was automatically regulated to provide 12 h of darkness and 12 h of light from 1600 to 0400 and 0400 to 1600 respectively The rats were fed *ad libitum* before the operation The food intake post-operatively was restricted to 12—13 g/day the average food consumption of normal rats of th[illegible] ze After hypothalamic surgery an automated food dispenser (Quarterman *et al* 1970) supplied th[illegible] daily portion [illegible]f food uniformly and continu[illegible]usly This was done in order to exclude adaptive metabolic effects due to mealfeeding and hyperphagia The diet contained 15 [illegible] protein 13 [illegible] fat and [illegible] carbohydrate made into a powdered mixture

*Su[illegible]g[illegible]al procedu[illegible]*

Stereotaxically guid[illegible]d lesi[illegible]ns (David Koph Instruments Mod l 900) were produced by electrolysis under Nembutal® anesthesia An anodal current (1 [illegible] mA 15 s) was pass[illegible]d through st[illegible]el electrodes of even th[illegible]ckness (0 4 mm) thor[illegible]ughly insulated by varnish except at the tip The stereotaxic co[illegible]rdinates used for destr[illegible]ying the ventromedial area of the hypothalamus were [illegible] 0 mm anterior to the inter aural line 0 6 mm lateral to the midline and 1 0 mm above the ventral flo[illegible]r of the skull As [illegible]ther experiments have revealed no effect of sham-operation upon the metabolism 2 da[illegible]s post-operatively (Løvø and Hustvedt in press) untreated animals were used as controls

*Histology*

The brain lesi[illegible]ns [illegible]f all ani[illegible]als [illegible]urgically treated were histologically examined All animals included in this study exh[illegible]bited [illegible]ymmetrical [illegible]ilateral lesions in the ventromedial hypothalamic area and no interruption [illegible]f the median eminence could be demonstrated

*Experimental procedure*

The insulin sensitivity of diaphragm and parametrial adipose tissue were assayed *in vivo* by studying the intraperitoneal acti[illegible]n [illegible]f exogen[illegible]us insulin (Rafaelsen *et al* 1965) The experiments were performed 2 days post-operatively after 16 h fasting and were always started between 0900 and 1000 In [illegible] experiment 2 groups of animals (9 with VMH lesions and

8 untreated controls) were given 2 0 μCi D glucose $^{14}C$ (U) (The Radiochemical Centre Amersham) contained in 2 5 ml saline by i p injection The solution contained 5 % bovine serum albumin (Sigma fraction V) and 0 1 mg glucose/ml Insulin was added to the saline solution in desired concentration to give each rat 0 1 1 0 and 10 0 mU respectively Bovine crystalline insulin (Novo lot nr 027667) was used The in vivo incubation was discontinued by decapitation of the animals after 2 h Blood was collected into heparinized tubes immediately placed on ice and the cells were separated from the plasma by centrifugation The hemi diaphragms and the parametrial fat pads were dissected free and immediately frozen on an aluminium block cooled by ethanol and dry ice within 2 min after the decapitation

*Analytical procedures*

The plasma glucose concentration was determined in 50 μl of plasma A commercial glucose oxidase reagent set was used (Glox® Kabi) The plasma urea concentration was assayed by using an enzymatic test kit supplied by Boehringer Mannheim The frozen hemi diaphragms were weighed and hydrolyzed for 30 min by boiling with 30 % KOH The glycogen determinations were always started immediately after the experiment Glycogen was precipitated over night at 4 C in 66 ethanol with 60 mM $Na_2SO_4$ After washing 2 times with 66 ethanol the precipitate was dried and dissolved in 2 ml aq dest. An aliquot of 1 8 ml was mixed with Insta-Gel® (Packard Instrument Comp Inc ) and counted in a Packard Tri Carb Liquid Scintillation Spectrometer Counting efficiency was calculated by means of the channels ratio method

The remaining 0 2 ml of solution was used for duplicate glycogen determinations This was performed by an all enzymatic method according to Adolfsson 1972 The glycogen was hydrolyzed to glucose by amyloglucosidase (Sigma grade II) and the liberated glucose was determined by the glucose oxidase method described Rabbit liver glycogen (Sigma Type III) was used as standard A separate standard curve was constructed during each run of the assay The parametrical fat pads were weighed and homogenized in chloroform methanol 2 1 The total lipids were isolated according to Folch *et al* (1957) and transferred to counting vials and taken to dryness The lipid material was weighed and 10 ml scintillation fluid (toluene containing 6 0 g PPO/l) was added Differences between groups were compared by Student s t test

## Results

Fig 1 shows the specific $^{14}C$ activity of parametrial lipids from animals with VMH lesions and their controls after *in vivo* incubation with D glucose $^{14}C$ (U) without exogenous insulin and with 0 1 1 0 and 10 0 mU respectively This demonstrates a more than 100 % increase in specific $^{14}C$ activity of the extracted lipids from the VMH lesioned group compared to the controls when no insulin was administred The difference is significant $p < 0\,001$ The lesioned animals exhibit a higher $^{14}C$

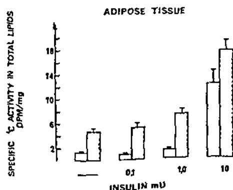



Fig 1 Specific $^{14}C$ activity of total extracted lipids (DPM/mg) from parametrial adipose tissue after i p injection of 2 μCi D glucose $^{14}C$ (U) together with 0 0 1 1 0 and 10 0 mU of insulin The open columns give the activity of the control groups (n = 8) while the hatched columns represent VMH lesioned animals (n = 9) The standard deviation of the means are given by the bars

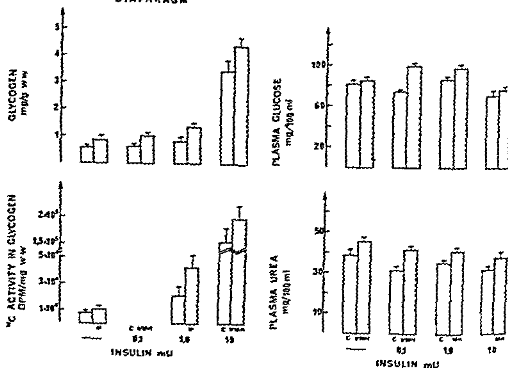



Fig. 2. Glycogen concentration (mg/g w.w., upper panel) and the $^{14}$C-activity in glycogen (DPM/mg w.w., lower panel) in the diaphragm after i.p. injection of 2 μCi D-glucose-$^{14}$C (U) together with 0.01, 1.0 and 10.0 mU of insulin. The open columns give the activity of the control groups (n = 8) while the hatched columns represent VMH lesioned animals (n = 9). The standard deviations of the means are given by the vertical bars.

Fig. 3. Plasma glucose concentration (mg/100 ml, upper panel) and plasma urea concentration (mg/100 ml, lower panel) after i.p. injection of 2 μCi D-glucose-$^{14}$C (U) together with 0.01, 1.0 and 10.0 mU of insulin. The open columns give the activity of the control groups (n = 8) while the hatched columns represent VMH lesioned animals (n = 9). The standard deviations of the means are given by the vertical bars.

incorporation than their controls also after stimulation with 0.1 and 1.0 mU of insulin. However, the difference was not significant following administration of 10.0 mU insulin. The figure also shows that only the highest dose of insulin (10.0 mU) stimulated the $^{14}$C incorporation in the control groups, while 1.0 mU gave rise to a significantly increased incorporation ($p < 0.025$) among the lesioned animals.

The glycogen concentration in diaphragms from VMH lesioned and control rats (mg glycogen/g wet weight) is shown in Fig. 2, upper panel. This demonstrates the stimulating effect of exogenous administered insulin on glycogen synthesis. The figure also shows increased levels of glycogen in the lesioned animals compared to the controls for all groups when exception is made for the groups given 10.0 mU of insulin. The differences are significant ($p < 0.05$). Only the highest insulin dose (10.0 mU) gave rise to any significant increase of the glycogen concentration. The

glycogen levels exhibit a 4 fold increase from 1 to 4 mg glycogen/g wet weight i lesioned animals as well as controls when compared to the unstimulated levels

The $^{14}C$ incorporation from D glucose $^{14}C$ (U) into glycogen in diaphragm is shown in Fig 2 lower panel A stimulating effect ($p < 0\ 001$) of insulin on the $^{14}C$ incorporation is manifest both in lesioned and control animals after injection of 1 0 mU Following administration of 10 0 mU of insulin a 10 fold increase in $^{14}C$ activity is observed The response seems to be of the same magnitude both in VMH lesioned and control animals

The mean plasma glucose concentrations of the different groups are shown in Fig 3, upper panel The values are within the normal range for fasting animals 70—100 mg/100 ml There are no significant differences between the mean glucose levels for animals given 10 0 mU of insulin and the groups given saline It may be pointed out that the mean plasma glucose concentrations in the groups of lesioned animals seem to be somewhat elevated compared to those of their controls The difference is significant for the groups given 0 1 and 1 0 mU insulin $p < 0\ 01$

The mean values for plasma urea concentration in the different groups are shown in the lower panel of Fig 3 It will be een that the urea levels in the VMH lesioned are significantly higher than in their controls $p < 0\ 01$

## Discussion

Increased lipogenesis is the most pronounced effect of the VMH lesion In the present experiments this is clearly demonstrated by the increased $^{14}C$ incorporation into total extracted parametrial lipids (Fig 1) Lipogensis is stimulated by exogenous insulin in both lesioned animals and their controls However only the highest in sulin dose (10 0 mU) had significant effect in the control group whereas 1 0 mU caused an increased incorporation in the lesioned animals The adipose tissue from these animals therefore semes to be more sensitive to the action of insulin than the controls This is contrary to what is expected if decreased insulin sensitivity is the primary cause of the hyperinsulinemia The glycogen concentration and the $^{14}C$ incorporation into glycogen in diaphragm is increased in the lesioned animals However the glycogen synthesis is stimulated to the same degree in lesioned and control animals (Fig 2)

These findings imply that the co existence of hyperinsulinemia and normo glycemia demonstrated in these animals are not due to decreased insulin sensitivity Our results thus support these presented by Frohman *et al* (1972) and extend their conclusion so as to include adult VMH lesioned animals 2 days post-operatively The conclusion is further supported by results from some recent experiments dealing with enzyme activities in muscle and liver after VMH surgery (Adolfsson *et al* submitted for publication)

The study was carried out to test whether a decrease in the activities of enzymes of importance for glucose utilization were included among the early metabolic alteratinos following VMH destruction No such decrease could be demonstrated

3 days post-operatively This is in contrast to the finding of decreased activity of glycolytic enzymes in muscle tissue from the genetic obese hyperglycemic mouse (obob) which is known to exhibit pronounced insulin resistance (Bray and York 1971 Adolfsson *et al* submitted for publication)

We have recently pointed out that the early onset of hyperinsulinemia post operatively indicates that VMH destruction may cause increased insulin secretion by a direct nervous or humoral action on the endocrine pancreas (Hustvedt and Løvø 1972 It has been reported that basal gastric secretion is elevated in VMH lesioned animals (Ridley and Brooks 1965) Therefore the possibility of a disturbance in the nervous control of gastro intestinal functions post-operatively merits further discusion This could modify insulin secretion directly by an altered activity in pancreatic nervous fibres and indirectly by altering the output of gastro-intestinal hormones The nervous participation in insulin secretion as well as the insulinotropic effect of gastro-intestinal hormones are well documented (Porte *et al* 1973 Dupre 1970

It seems difficult however to account for the normal blood glucose levels during fasting in presence of hyperinsulinemia and normal insulin sensitivity in terms of elevated insulin secretion only In our opinion the most likely reason for this finding is a greater output of glucose from the liver The data presented give no direct evidence for changes in liver metabolism but the elevated levels of plasma urea indicate increased gluconeogenesis This is further supported by the increased amino acid catabolism of lesioned animals demonstrated by nitrogen balance studies (Holm *et al* in press)

These results raise the question of whether the secretion of glucagon as well as that of insulin may be altered in VMH lesioned animals Increased release of glucagon would promote gluconeogenesis and accelerate the glucose release from glycogen Possibly this could produce in concert with the lipogenic effect of elevated insulin secretion the accumulation of body fat at the expense of protein which characterizes the VMH syndrome Current evidence suggests that stimuli affecting insulin release as nervous stimulation and gastrointestinal hormones also influence pancreatic glucagon secretion (Porte *et al* 1972 Iversen 1971)

However there also remains the possibility that VMH destruction could produce changes in liver metabolism by alterations of the activity in hepatic nervous fibres Recent studies have shown that enzymes implicated in hepatic glycogen and amino acid metabolism are under the influence of the autonomic nervous system (Shimazu and Fujimoto 1971 Black and Axelrod 1971)

In summary we suggest that the profound metabolic effects of VMH destruction is caused by a disturbance in the nervous control of gastro-intestinal functions This may lead to alterations in the endocrine pancreatic secretion and possibly also in liver metabolism

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# Distribution of Carbonic Anhydrase in the Frog Nephron

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## Abstract

LÖNNERHOLM G and Y RIDDERSTRÅLE *Distribution of carbonic anhydrase in the frog nephron* Acta physiol scand 1974 *90* 764—778

The distribution of carbonic anhydrase in the kidney of the frog (Rana temporaria) was studied by the histochemical method of Hansson using light and electron microscope In all cells showing enzyme activity the staining deposits seemed to be diffusely distributed without any consistent local accumulation The proximal tubule contained no or little enzyme Some part of the distal tubule showed high enzyme activity whereas the remainder was inactive In the initial collecting tubule a mosaic pattern of stained and unstained cells was found the stained cells being identical with the so called canaliculi cells The collecting duct contained some stained cells too The renal corpuscle the neck and the intermediate segment were inactive Apparently the enzyme is less widely distributed along the kidney tubules of the frog compared to the tubules of man and rat where enzyme activity is found in the convoluted part of the proximal tubule the whole distal tubule and in many cells in the collecting duct These differences in enzyme distribution as well as certain physiological data might indicate differences in the localization of the urinary acidification between the frog and these mammals

Considerable amounts of the enzyme carbonic anhydrase (EC 4 2 1 1) seem to be present in the kidneys of all vertebrates except seagoing fishes (see Maren 1967 a) Its importance for urinary acidification is proposed by experiments with inhibitors of the enzyme (see Maren 1967 b 1969) For the discussion of the role of the enzyme it seems necessary to know where along the kidney tubules it is present This matter has so far been studied in detail only in a few mammalian species in the rat by the histochemical method (Lönnerholm 1971) in the dog by assaying microdissected parts of the kidney (Mattenheimer *et al* 1964) and in man by both kinds of methods (Mattenheimer *et al* 1970 Lönnerholm 1973) These studies showed a distribution of the enzyme in the proximal and distal tubules as well as in the collecting duct This indicates that the enzyme is present in those parts of the kidney tubules where there is a postulated role for it (Pitts 1968 Rector 1971)

In the frog kidney the sites of the urinary acidifying processes and the role of the enzyme for these processes are not so well known Single studies have suggested that the urinary pH is lowered already in the proximal tubule (Ellinger and Hirt 1929

1930) whereas others demonstrated that the hydrogen ion secretion is restricted to the more distal parts of the kidney tubules (Richards 1929 Montgomery and Pierce 1937 Walker 1940) The latter findings would indicate that the frog differs from the rat where the proximal convoluted tubule is known to secrete large amounts of hydrogen ions (see Rector 1971) Moreover segments with markedly different abilities to secrete hydrogen ions have not been found in the distal tubule of the rat kidney but may exist in the frog (Montgomery and Pierce 1937) The purpose of the present work was to study the distribution of carbonic anhydrase in the kidney of the frog (*Rana temporaria*), mainly by a recently introduced histochemical method in an effort to clarify the sites of the urinary acidifying processes in this species This communication also describes the adaptation of the histochemical method for electron microscopic observations

## Materials and Methods

Male and female frogs (*Rana temporaria*) weighing 25—40 g were used They were delivered from Stockholms Biologiska Laboratorium AB Akersberga, Sweden and kept in a container with running tap water at 10 C for one or several weeks before use The frogs were fed raw pork liver once a week using a pair of forceps

### *Histochemical method*

25 animals were studied during September to December

*Preparation of tissue* Unfixed kidneys were rapidly removed after decapitation of the frogs and were immediately frozen in isopentane cooled with liquid nitrogen In some experiments the kidneys were perfused with isotonic saline (0 6 % NaCl) before removal and freezing These frogs were anesthetized with urethane 2 g/kg b wt. injected into the dorsal lymph sac 10—15 ml of 0 6 % NaCl was then infused through a needle introduced into the heart after opening the sinus venosus

*Fixed* kidneys were obtained from urethane anesthetized frogs using 2 5 % glutaraldehyde in 0 067 M phosphate buffer at pH 7 4 as fixative agent The glutaraldehyde was prepared from a 50 % stock solution by a one stage vacuum distillation (Anderson 1967) 10—15 ml of fixative was infused through a needle inserted into the anterio abdominal vein in the caudal direction This vessel communicates with the renal portal veins which together with the renal arteries form the dual blood supply to the frog kidneys (for anatomical details see Holmes 1934) In some experiments the fixative was infused into the heart as described above After removal the kidneys were divided into 3—4 pieces and immersed in fixative for 1—2 h at 4 C briefly rinsed in 0 6 % NaCl and frozen

Frozen tissues were kept in small plastic bags at −70 C for days or weeks before use

*Staining procedure for light microscopy* The sections were stained for carbonic anhydrase activity according to the method of Hansson (1967 1968) In this method a complex containing cobalt and phosphorous is formed at sites of carbonic anhydrase activity By adding ammonium sulfide the complex is converted to a black precipitate probably containing CoS which is easily visualized

The specificity of this method has recently been confirmed by Rosen and Musser (1972) and in this laboratory (Lonnerholm in preparation) in response to the criticism by Muther (1972)

The staining procedures were as follows

Kidney sections were cut at −20 C

4—8 μm sections of *unfixed* tissue were thawed on a TH WP Millipore® filter (25 μm thick, pore size 0 45 μm Millipore filter corporation Bedford Mass USA) and then incubated as described below

Sections of *fixed* tissue were handled in one of two ways

1) 4 μm sections were thawed on Millipore® filters

2) 8—10 μm sections were collected in a Petri dish containing cold 0 13 M sucrose in

0.033 M phosphate buffer at pH 7.4 and were thereafter transferred to the incubation medium within a few min.

Free floating sections as well as sections on their Millipore® filters were floated on the surface of the freshly prepared incubation medium in a 9.5 cm Petri dish. To prevent the free floating sections from disintegrating in the slightly effervescent incubation medium the non-ionic detergent Tween 20® was added to a final concentration of 1/100 000 (v/v). The sections on Millipore® filters were allowed to equilibrate with the incubation medium for 10 min under a 100 % $CO_2$ atmosphere (see Hansson 1968). The medium contained $CoSO_4$ $1.75\times10^{-3}$ M, $H_2SO_4$ $5.3\times10^{-3}$ M, $NaHCO_3$ 0.157 M and $KH_2PO_4$ $11.7\times10^{-3}$ M and its volume was 57 ml. Incubation times of 1—12 min were found to be most suitable for the frog kidney tissue. The sections were then rinsed for 3 min in 0.6 % NaCl buffered with phosphate ($4.5\times10^{-4}$ M, pH 5.9) before transfer to the blackening 0.5 % $(NH_4)_2S$ solution. The sections were finally rinsed in 0.6 % NaCl. All solutions were kept at room temperature.

Sections with and without counterstaining with haematoxylin and eosin were prepared. Free floating sections were dehydrated through graded concentrations of ethanol and xylene and mounted with Canada balsam. Unfixed sections on their Millipore® filters were dehydrated in 95 % ethanol, absolute n-propanol, xylene and absolute n-propanol (1:1)-xylene and mounted in Canada balsam.

Microphotographs were taken on Kodak Panatomic X film using a Zeiss standard microscope GFL with a Zeiss photo attachment.

*Procedures for electron microscopy.* Kidneys fixed by perfusion through the abdominal vein were used.

10 μm thick, free floating sections were stained for carbonic anhydrase activity as described above. Those taken for electron microscopy were close to those taken for light microscopy. After the final rinsing in 0.6 % NaCl the sections were collected on small pieces of filter paper and remained there through the whole embedding procedure. The wet pieces of filter paper with their sections were placed on the walls of glass or plastic bottles with tight caps. They adhered well to the walls as long as they were wet. A small amount of 2 % $OsO_4$ solution was put into the bottle and the pieces were gently pushed down until the edge of the paper reached the solution. After fixation for half an hour the $OsO_4$ solution was drawn off and the sections were dehydrated by increasing concentrations of ethanol and finally propylene oxide. Infiltration of the sections was performed in a mixture of propylene oxide and epon for 2 h and then in pure epon for 1 h (Luft 1961). The sections were embedded in fresh epon in aluminium capsules to get flat embeddings. The specimens were laid with the section facing the bottom of the capsule.

Parts of the kidney tubules for ultrathin sectioning were selected by studying the light microscopic preparations and then finding the corresponding part of the epon embedded section. The trimming was done on a LKB Pyramitome so that ultrathin sections either parallel to the original sectioning direction or transverse to it were obtained. During the trimming survey sections were taken and studied in a phase contrast microscope to get the exact position for the ultrathin sectioning, which was performed with a diamond knife on a LKB Ultrotome I. Some ultrathin sections were stained with aqueous uranyl acetate, whereas others were left unstained. The sections were examined in a Hitachi HU 11 E electron microscope and micrographs were taken on Scienta plates with emulsion 23 D56.

*Biochemical method*

These experiments were performed in December. Kidneys perfused with 0.6 % NaCl as described above were removed and decapsulated. They were generally pale and appeared free from blood. Incompletely perfused kidneys were discarded. The kidneys were divided longitudinally into a ventral and a dorsal part of about the same thickness. Fresh kidneys were similarly divided using a modified Stadie-Riggs slicer or frozen by $CO_2$ and cut by a Leitz bench cryotome. The latter method permitted better control of the cutting procedure.

A tight fitting Teflon® plunger in a glass tube was used for homogenization and the homogenates were diluted 1:10 in cold glass-distilled water containing $10^{-3}$ M EDTA (sodium salt) to protect the enzyme from inactivation. The kidneys of 7—8 frogs were pooled to give enough tissue for the assay.

The carbonic anhydrase activity of the homogenates was determined within a few hours by the changing pH method (Philpot and Philpot 1936). $CO_2$ is bubbled at constant rate through a reaction vessel containing phenol red. A standard amount of carbonate is then added, causing the indicator to change color. The hydration of $CO_2$ forms $H_2CO_3$ which neutralizes the added buffer base and causes the indicator to return to the original (acid) color. One enzyme unit is defined as the activity required to reduce the reaction time by half

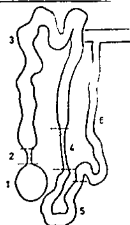



Fig. 1 Diagram of the frog kidney tubules 1) Bowman's capsule 2) neck 3) proximal tubule 4) intermediate segment, 5) distal tubule 6) initial collecting tubule 7) collecting duct.

The activity of a homogenate was calculated from 1—2 determinations on each of 3—4 dilutions of the homogenate. The assays were performed at 0 2 C

The hemoglobin concentration in the kidney homogenates was determined by a peroxidase method (Bing and Baker 1931) Whole blood was also sampled from the frogs and its carbonic anhydrase activity was assayed

*Nomenclature*

The course of the frog kidney tubules is shown in Fig 1 At least seven different parts can be distinguished. The figures in brackets refer to numbers of Fig 1 The *capsule of Bowman* (1) is connected with the proximal tubule by the *neck* (2) lined by low ciliated cells The *proximal tubule* (3) is situated almost exclusively in the dorsal part of the kidney It is easily recognized by its high brush border The cells are rich in mitochondria, have large apical vacuoles and the basal cell membrane forms irregular infoldings. The fourth part of the kidney tubules is structurally similar to the neck and is here called the *intermediate segment* (4) It is followed by the *distal tubule* (5) which consists of cells with numerous mitochondria and deep basal cell membrane infoldings The apical cell surface has short microvilli The convolutions of this segment are mainly situated at the ventral surface of the kidney The *initial collecting tubule* (6 consists of two cell types which are here called light cells and canaliculi cells. The light cells are apparently devoid of structural peculiarities The canaliculi cells have an intracellular canaliculus which opens into the tubular lumen and they are very rich in mitochondria and often have multivesicular bodies (Wiegert and Ekberg 1903 a, b Geyer and Linss 1964 Bargmann and Welsch 1972) Generally they have a triangular form with a broad surface often somewhat bulging and a narrow apical surface This fact to some extent gives them the appearance of lying on the outside of the tubule. The first part of the initial collecting tubule is somewhat convoluted and mingles with the most dorsal of the convolutions of the distal tubule The latter part of it runs almost straight towards the dorsal surface of the kidney where it joins the *collecting duct* (7 This duct consists mainly of light and rather empty cells but a few canaliculi cells may occur The collecting ducts extend transversely across the dorsal surface of the kidney and empty in the ureter at the lateral edge of the kidney

## Results

### *Histochemical method*

*Light microscopy* The results of the staining for carbonic anhydrase activity in the different parts of the kidney tubules are summarized in Table I Similar results were obtained with fixed and unfixed kidneys and the way of preparing the tissue

TABLE I Histochemical staining for carbonic anhydrase activity in different parts of the frog kidney tubules + = positive staining − = negative staining (+) = weak and inconsistent staining

| Kidney region | Staining |
|---|---|
| Glomerulus | − |
| Bowman s capsule | − |
| Neck | − |
| Proximal tubule | (+) |
| Intermediate segment | − |
| Distal tubule | |
| stained part | + |
| unstained part | − |
| Initial collecting tubule | |
| light cells | − |
| canaliculi cells | + |
| Collecting duct | |
| light cells | − |
| canaliculi cells | + |

(see Methods) did not influence the staining No differences were found between cranial and caudal parts of the kidney or between males and females Animals sacrificed during September to December gave similar results

The glomerulus Bowman s capsule the neck the intermediate segment and the renal interstitium were always unstained Fig 2 3

The cells of the *proximal tubule* were not distinctly stained In some kidneys they




Fig 2 Frog kidney stained for carbonic anhydrase activity No counterstain Fixed 8 μm thick section incubation time 12 min DS = dorsal surface VS = ventral surface Some distal tubules situated at the VS are heavily stained. Single cells in initial collecting tubules and collecting ducts (the latter are seen close to the DS) are also stained ×65

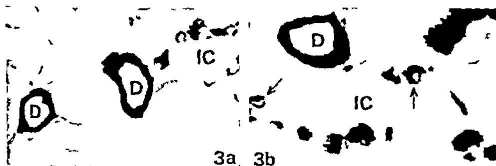



Fig 3 Frog kidney ventral part, stained for carbonic anhydrase activity No counterstain Fixed 4 µm thick sections incubation time 12 min D = distal tubule IC = initial collecting tubule with stained and unstained cells Some of the stained cells show a clearly visible intracellular canaliculus (3b arrows) The unstained tubules are probably identical with distal tubules 3a ×2?0 3b ×540

were completely unstained even after an incubation time of 12 min Fig 2 In other kidneys however weak staining could be observed This staining was distributed over the whole cells including cytoplasm nucleus and brush border region

Part of the *distal tubule* showed high enzyme activity In this part the whole cells where heavily stained Fig 2 3 and the staining was clearly visible already after short incubation times of 1—3 min The stained tubules were found in the ventral part of the kidney However other tubules in this region were completely unstained even after incubation times of 12 min The localization and structure of the different tubules indicated that they were all parts of the distal tubule which was confirmed by the electron microscope Unstained transections of the distal tubule seemed to be more common than stained ones suggesting that only a minor part of the distal tubule contained the enzyme

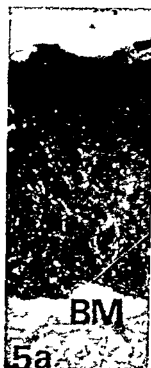
In the *initial collecting tubule* many cells were completely unstained whereas others were distinctly stained after incubation times of 3—6 min Fig 2 3 The unstained cells appeared to be more numerous than the stained ones With fixed kidneys many of the stained cell showed an intracellular canaliculus clearly visible in the light microscope Fig 3 b arrows

The *collecting duct* also contained stained and unstained cells Fig 2

*Control experiments* When the carbonic anhydrase inhibitor acetazolamide (Diamox® American Cyanamid Company Pearl River N Y USA) at 10 M was added to the incubation medium all sections remained completely unstained A lower concentration 10⁶ M gave a much delayed and weakened staining reaction The inactive control substance Cl 13 850 a N³ t butyl analogue of acetazolamide (American Cyanamid Company) at 10⁴ M did not interfere with the staining

*Electron microscopy* When studying ultrathin sections cut transversely through the original 10 µm thick sections it was found that the staining deposits were very compact at the surface which had been in contact with the air during the incubation Fig 4 The intensity of the staining decreased gradually through the sections and the lower part was left unstained This distribution of the staining is



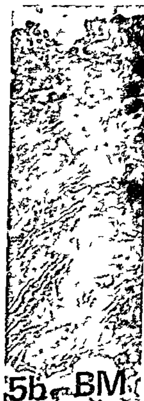




Fig 4 Ultrathin section cut transversel to the original sectioning direction of a 10 μm thick ection Incubation time 9 min Parts of a stained cell is shown The precipitate is hea y close to the upper surface (US) and gradually decreases through the section The lower part of the section (not hown) is completely unstained ×35 000

Fig 5 Distal tubular cells BM = basal membrane Ultrathin sections cut parall l to th original se tioning direction 5a cut through region with hea y staining close to th upper surface 5b hows region deeper in the section where only traces of the precipitate are found Incubation time 9 min Uranyl acetate 5a 5b×10 000

due to the fact that specific staining at carbonic anhydrase sites can occur only where loss of the reaction product $CO_2$ is not rate limiting (Hansson 1967)

When the original 10 μm thick ections were cut transversely the ultrathin sections showed only small parts of the cells In ultrathin sections cut parallel to the original sectioning direction complete transections of tubules could be studied and uch ections were generally used In the surface region the compact precipitate often completely obscured the ultrastructure of the stained cells In order to get a satisfactory identification of the stained cells ultrathin sections had to be cut at




Fig. 6 Distal tubule with all cells stained Ultrathin section cut parallel to the original sectioning direction Incubation time 9 min Uranyl acetate ×7000

first in the surface region of the specimen showing the sites of heavy staining Fig 5a and then at lower levels until the ultrastructure of the stained cells was discernible Fig 5b At the upper surface of the sections it was sometimes found that the precipitate extended somewhat beyond the boundaries of the stained cells Deeper in the sections the deposits appeared limited to the primarily stained cells Fig 7 The precipitate seemed to make the tubules brittle and heavily stained tubules were often broken within the section Fig 6 whereas neighbouring unstained tubules were better preserved

The electron microscopic study was concentrated on those parts of the kidney tubules which showed distinctly stained cells in the light microscope *i e* the distal tubule and the initial collecting tubule

In some tubules usually found in the ventral part of the kidney all cells of the tubular transection were intensely stained Fig 6 By serial sectioning (see above) they could with certainty be identified as *distal tubular* calls However in many tubular transections also undoubtedly belonging to the distal tubule the cells were without any trace of staining In well preserved frog kidneys several segments can be distinguished within the distal tubule (Ridderstrale unpublished observations) The presently available material did not allow such a distinction and thus it was not possible to decide where along the distal tubule the stained cells were situated

Some tubular transections showed single stained cells among unstained ones



Fig 7 Canaliculus cell with heavy precipitate A neighbouring unstained cell is seen at the top At lower left the lumen of a blood vessel Ultrathin section cut parallel to the original sectioning direction Incubation time 9 min Uranyl acetate ×6300

Fig 7 They were all found to belong to *initial collecting tubules* and with regard to either form and position or/and ultrastructure each stained cell could be identified as a canaliculus cell The light cells were always unstained

The precipitate was diffusely distributed within the stained cells distal tubular as well as canaliculi cells Fig 5—7 without consistent concentration to any cell organelle or to cellular membranes

*Biochemical method*

Frog kidneys divided into a ventral and a dorsal part were assayed for carbonic anhydrase activity after homogenization (Table II) The ventral part was several times more active than the dorsal part Small amounts of hemoglobin were found in the homogenates Determinations of enzyme activity in whole blood samples revealed that less than 3% of the total enzyme activity in the kidney homogenates was due to contaminating erythrocytes

In one experiment homogenates of the two parts of the kidney were incubated in 5% (v/v) of the non ionic detergent Tween 20® for 5 min a procedure which has been shown to increase the activity in lens homogenates (Wistrand and Rao 1968) No change in activity was found however

TABLE II Carbonic anhydrase activity in pooled frog kidney homogenates Enzyme units/g wet weight of tissue Each figure represents one homogenate Values were corrected for the activity of contaminating erythrocytes

| | Ventral part | Dorsal part |
|---|---|---|
| A Fresh kidneys | 230 | 100 |
| B Frozen kidneys | 250 | 50 |

The $I_{50}$ i e the molar concentration which inhibits 50 % of the enzyme activity for acetazolamide against this frog kidney enzyme was 2—3×10 $^{8}$ M which is very similar to that for human and rat kidneys (Maren and Ellison 1967 Maren 1969)

## Discussion

1 *Carbonic anhydrase in the frog kidney previous and present findings*

The present histochemical findings show high enzyme activity in some cells of the distal tubule the initial collecting tubule and the collecting duct The findings in the proximal tubular cells however are difficult to interpret since the staining was absent in some kidneys and only weak in others It could be due to low enzyme activities close to the detection threshold of the histochemical method

Rosen (1972 a) who also used the Hansson method found enzyme activity along the entire length of the proximal tubule of the toad (*Bufo marinus*) He did not study the distal tubule but in the collecting duct which probably includes also the segment named initial collecting tubule in the present study he found the same alternating pattern of densely stained and unstained cells as seen here in the frog The difference between the frog kidney on the one hand and the toad and mammalian (see below) kidney on the other with respect to carbonic anhydrase activity in the proximal tubule is intriguing and should be further investigated

The present histochemical findings explain the large difference in enzyme activity between the ventral and the dorsal halves of the frog kidney (Table II) The ventral half showing an activity similar to that of homogenates of mammalian kidney cortex (see Maren 1967 b) contains the histochemically highly active distal tubular cells and some of the canaliculi cells The dorsal half contains some active cells from the initial collecting tubules and the collecting ducts but consists mainly of proximal tubules with no or low activity

The technique used to cut the kidney into a dorsal and a ventral half will obviously influence the ratio of enzyme activity between the halves This might possibly explain why Maren (1959) found similar activities in both halves of the kidney of the frog (*Rana clamitans*) although it cannot be ruled out that it was due to species differences Such differences or seasonal variations might explain why Maren found relatively low activities whereas Yata (1961) found that homoge

whole frog kidneys (*Rana catesbeiana*) were as active as those of whole dog kidneys

In the present study the distribution of the enzyme activity was outlined by the light microscope and the electron microscope was used to ascertain the identification of the stained cells The intracellular distribution of the staining deposits was also studied by the electron microscope The precipitate was diffusely distributed in all stained cells apparently without consistent accumulation to any cell organelle However it is not clear at present if this represents the true intracellular distribution of the enzyme or if diffusion artifacts might have occurred

In kidney homogenates from man (Wistrand and Wahlstrand personal communication) dog and rat (Baumann 1961 Maren and Ellison 1967) and frog (*Lonnerholm* preliminary data) carbonic anhydrase activity is found chiefly in the supernatant fraction However some is also found in the microsomal and mitochondrial fractions (not studied in the frog kidney) Whether this latter activity is due to an enzyme bound to the membranes of these organelles or a cytoplasmic enzyme adhering to these membranes is not known

There are only a few previous histochemical studies of carbonic anhydrase activity at the ultra structural level In mouse liver cells (Yokota 1969) and rat stomach parietal cells (Cross 1970) the stain deposits were found close to cell membranes However in turtle and toad urinary bladder cells (Rosen 1972 b) and some cells of the retina of several vertebrate species (Musser and Rosen 1973) the precipitate was found mainly in the cytoplasm Different methods were used in these studies Cross (1970) used Hausler s method, Rosen (1972 b) and Musser and Rosen (1973) Hansson s method and Yokota (1969) a modification of Hansson s method

The presently used method for staining and preparation of the tissue for electron microscopy was found to have certain drawbacks The deposits were restricted to the upper surface of the original 10 µm thick sections Consequently the amount of staining found in an ultrathin section depended not only on the enzymatic activity but also on the region within the original section from which the ultrathin section had been taken Negative staining could simply be due to that the ultrathin section had been taken from a deep region where no precipitation had occurred

These circumstances made the interpretation of the findings difficult and necessitated that series of ultrathin sections were cut from each original section including some ultrathin sections from the region close to the upper surface These difficulties apparently exist also with the other histochemical methods for carbonic anhydrase used with electron microscopy as Cross (1970) and Rosen (1972 b) reported uneven distribution of the precipitate within stained cells

## 2 *Role of carbonic anhydrase in the frog kidney*

In the mammalian kidney high carbonic anhydrase activity is found inside the proximal and distal tubular cells (Mattenheimer *et al* 1964 1970, Lonnerholm 1971, 1973 Rosen 1972 a) where it provides a steady supply of hydrogen ions to

the secretory mechanisms by catalyzing the hydration of CO (Pitts 1968 Ke 1971) Micropuncture (Rector *et al* 1965) and histochemical (Lonnerholm 19 1 studies in the rat indicate that the enzyme is also present in the brush border of the proximal tubular cells It has been suggested (Rector *et al* 1965) that this enzyme catalyzes the dehydration of carbonic acid formed in the tubular fluid by the reac tion of secreted hydrogen with filtered bicarbonate

In the frog administration of inhibitors of carbonic anhydrase produces similar changes in urinary composition as those seen in mammals *i e* increased urinary pH and an increased output of bicarbonate sodium potassium and fluid (Hober 1942 Yoshimura *et al* 1961) Thus carbonic anhydrase appears to have an important role in the urinary acidification also in the frog However it is not clear at present to what extent the *proximal tubule* of the frog participates in hydrogen ion secre tion The *observations of Ellinger and Hirt (1929 1930) using fluorescein as an* indicator suggested that the pH of the proximal tubular fluid can be lower than that of plasma in the frog (*R esculenta*) However Richards (1929) and Mont gomery and Pierce (1937) found unchanged urinary pH along the proximal tubule of the frog (*R pipiens*) using injection of phenol red Ammonia did not appear in detectable amounts in the proximal tubular fluid of *R pipiens* and *R catesbeiana* (Walker 1940) The frog would then differ from the rat where about 70 % of ex creted ammonia is secreted in the proximal convoluted tubule (Glabman *et al* 1963 Hayes *et al* 1964) and tubular fluid pH is depressed to 6 8—7 0 reflecting a re absorption of 70—90 % of filtered bicarbonate (Gottschalk *et al* 1960 Vieira and Malnic 1968)

The present findings cannot clarify the role of the frog s proximal tubule in the urinary acidification since small amounts of the enzyme may or may not be present However the histochemical findings differ clearly from those of the rat and other mammals (see above) which supports the idea that the pattern of urinary acidifica tion may differ between these species The role of the proximal tubule in the frog should be further investigated by micropuncture techniques to solve this problem

In the *distal tubule* of the frog kidney both physiological experiments and the presence of carbonic anhydrase indicate secretion of hydrogen ions Thus in experi ments with phenol red Montgomery and Pierce (1937) found an abrupt color change from red (pH 7 4 or more) to yellow (7 0 or less) when a certain point of the distal tubule was reached By injection of phenol red into distal tubules when urinary flow was stopped they showed that rapid acidification of the urine took place only in a short segment of the distal tubule This segment constituted about one fifth of the total length of what Montgomery and Pierce called distal tubule which apparently includes also the segment named initial collecting tubule in the present study It was situated somewhat nearer the distal than the proximal end Walker (1940) found that ammonia was not added to the tubular fluid until the middle part of the distal tubule was reached Apparently this segment at least partly coincides with the acidifying segment of Montgomery and Pierce The present findings of high concentrations of carbonic anhydrase within some part of

whole frog kidneys (*Rana catesbeiana*) were as active as those of whole dog kidneys

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along the nephron Very high concentrations of inhibitor may be necessary to produce an effect if only a minor fraction of uninhibited enzyme suffices for the physiological function under study However some cells may have relatively little enzyme to spare and thus respond to considerably less inhibitor In other words the dose response curves for different cells along the nephron may differ considerably

The present work was supported by grant 04X 2874 from the Swedish Medical Research Council by Regnellska donationsfonden and by grant 3285 1 from the Swedish Natural Science Research Council

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Acta physiol scand 1974 90 779—788

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# Glycogen Storage and Glycogen Synthetase Activity in Trained and Untrained Muscle of Man

By

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Received 9 November 1973


## Abstract

Piehl K S Adolfsson and K Nazar *Glycogen storage and glycogen synthetase activity in trained and untrained muscle of man* Acta physiol scand 1974 90 779—788

Bilateral biopsies from one leg trained subjects were analysed for glycogen content (G) total glycogen synthetase activity (GST) synthetase I form (GSI) and hexokinase (HK) Histochemical estimations were made of G GST and GSI in slow twitch (ST) and fast twitch (FT) muscle fibres Samples from the trained (T) and the untrained (U) legs were taken before and after acute exhaustive exercise and at different intervals during a 46 h recovery period when a carbohydrate enriched diet was given Prior to exercise G levels were higher (32 %) in T leg than in U leg Throughout the experiment T leg showed higher activity for GST (35 %) and HK (18 %) In both U and T legs GSI activity increased (from 16 to 48 %) after exercise The increase in GSI was histochemically found mainly in ST fibres in both T and U leg while the staining intensity for GST was similar in ST and FT fibres During the recovery period G was restored and GSI gradually declined to resting values The rate of synthesis was the same in T and U legs and in the two fibre types


Physically well trained humans have higher glycogen levels in their muscles (Gollnick *et al* 1972 a 1973 a) If this phenomenon is due only to training or partly to an uncontrolled increase of carbohydrates (CHO) of the diet has not conclusively been shown There are very few studies on the influence of training on the enzymes involved in glycogen metabolism in human muscle The enzyme glycogen synthetase (GS) (EC 2 4 1 11) is considered to be rate limiting in glycogen synthesis (for ref see Adolfsson 1972) and consists of at least two interconvertable forms the less active D form dependent on glucose 6 phosphate (G 6 P) for its activity, and the more active I form independent of this co-factor The enzyme hexokinase (HK) (EC 2 7 1 1) may also be of importance in the regulation of glycogen synthesis

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TABLE I Physical characteristics of the subjects T = trained U = untrained For further information see text

| Subject | Age years | Weight kg | Max $V_{O_2}$ l/min | | | SDH µmol × g⁻¹ × min⁻¹ | |
|---|---|---|---|---|---|---|---|
| | | | two leg | T leg | U leg | T leg | U leg |
| KG | 21 | 67 | 4 5 | 3 6 | 2 8 | 5 8 | 4 7 |
| SR | 22 | 72 | 3 5 | 2 8 | 2 6 | 4 1 | 3 9 |
| JG | 24 | 60 | 3 4 | 2 9 | 2 7 | 6 1 | 3 2 |
| SH | 23 | 69 | 3 9 | 3 6 | 2 8 | 4 0 | 3 0 |
| BS | 23 | 69 | 4 9 | — | — | 6 1* | — |
| NM | 27 | 67 | 3 2 | — | — | — | 3 5** |

* Both legs well trained
** Both legs untrained

Data indicating that the glycogen (G) depletion pattern after exercise was different in the two fibre types of human skeletal muscle the slow twitch (ST) and the fast twitch (FT) fibres are available (Costill *et al* 1973 Gollnick *et al* 1973 b 1973 c Gollnick Piehl and Saltin 1974 Gollnick *et al* 1974) The aim of this study was to further study this hypothesis and to analyse the effects of training on the glycogen synthetizing capacity of the muscle

A one leg training model was used so each subject could serve as his own control After completion of the training programme muscle biopsies were taken before and after an acute exhaustive exercise For 46 h following the exercise a diet rich in CHO was given and the recovery was studied by means of repeated biopsies G levels and GS and HK activity were determined biochemically Histochemical analyses were also made in order to identify differences between the two fibre types

## Subjects

Six male subjects were studied Their physical characteristics are presented in Table I Subject NM was untrained BS well trained and the other 4 had completed a programme in which one leg was endurance trained by pedalling on a bicycle ergometer for 1/2 h three times a week for at least 6 weeks The load was adjusted to produce a heart rate exceeding 170 beats per minute 2 of the subjects trained their left legs and the other 2 their right legs Table I also presents values for maximal oxygen uptake (max $V_{O_2}$) and succinate dehydrogenase (SDH) activity These parameters are commonly used as indicies for the degree of training (Gollnick *et al* 1972 a 1973 a)

## Methods

Muscle samples were taken from the lateral portion of M quadriceps femoris using a needle biopsy technique (Bergström 1962) Two separate biopsies sometimes had to be taken in order to obtain a sufficient amount of tissue the second sample was taken from the same site as the first one to minimize the influence of regional variations within the muscle

The muscle sample was divided into 3 parts used for estimation of GS activity histochemical analyses and analyses of glycogen content and HK activity The muscle piece for GS activity determination was quickly weighed on an electrobalance frozen and stored in dry ice

for not more than 2 days In order to correct for water evaporation a stop w started when the biopsy was taken the weight was read three times every 30 s a extrapolated back to zero time GS activity was determined in a final dilution of 1 80 using a modified version of the method described by Thomas Schlender and Larner 1( (f r details see Adolfsson 1973 a) Radioactivity incorporated into glycogen from UDP glucose $^{14}C$ was measured in the absence and presence of 10 mM G 6 P Incorporation in th presence of this co-factor reflects total enzyme activity (I+D form) and is expressed in $\mu$m l of glucose incorporated into glycogen per minute and g of wet tissue In the absence of G-6-P only the active I form (GSI) was measured It is expressed as a percentage f total synthetase activity The part of the biopsy sample used for histochemical an l as mounted on pecimen holders in O C T embedding medium (Ames Tissue Tek) and frozen in isopentane cooled in liquid nitrogen and cut in a cryostat at −20 C (Gollnick *et al* 1 ) The two major fibre types were identified on the basis of the staining intensity f myo fibrillar ATPase (Padykula and Herman 1955) and DPNH-diaphorase (Novikoff Shin and Drucker 1961) as previously described (Gollnick *et al* 1972 a) Histochemical analyses of GS activity were carried out in the absence and presence of G 6 P to provide an indication of I form activity and total (I+D form) activity respectively as described by Takeuchi and Glenner (1963) The periodic acid Schiff (PAS) reaction was used to demonstrate th distribution of glycogen in fibres (Pearse 1961) PAS staining intensity was subjectively rated under a light microscope in four different categories dark moderate light and negative (Gollnick *et al* 19?? b) The remainder of the sample was frozen in liquid nitrogen and stored at −80 C until subsequently analysed for total glycogen content (Karlsson Diamant and Saltin 1970) Glycogen is expressed as mmol glucose units × kg $^{-1}$ wet tissue The activity of HK and of SDH was analysed according to Lowry and Passonneau (1973) and Cooperstein Lazarow and Kurfess (1950) respectively and expressed in $\mu$mol of substrate converted per minute and g wet tissue

The diet was composed so that 60 % of the kcal was derived from CHO and corresponded to 35 kcal per kg b wt and day Total caloric intake during the day was 3600—4000 kcal (1 kcal = 4 19 kJ) Breakfast amounted 300—500 kcal lunch 1200 kcal dinner 1800 kcal and snacks 300—500 kcal

## Procedure

During the last week of the training programme $\dot{V}o_2$ max and SDH activities were determined f r each subject 3 days after the last training session the subjects came to the laboratory at 8 a.m Muscle samples were then taken at rest and endurance exercise was performed on a bicycl ergometer at a load required approximately 70—80 of the subjects maximal aerobic power in addition short supramaximal work bouts were performed to the point of exhaustion As reported earlier combined exercise of this type produce glycogen depletion in most fibres (Piehl 1973 Gollnick Piehl and Saltin 1974) The first post-exercise biopsy was taken 5 min after completion of exercise to permit the GS enzyme to recover from its inhibition during exercise (Stanelloni and Piras 1969 Adolfsson 1973 b) During the following 46 h the CHO enriched diet was given and the subjects were not allowed to participate in any physical activity except slow walking During the first 10 h samples were taken 3 h after food intake (Fig 1) The two last samples were taken before breakfast at the 22nd and 46th h respectively The entire procedure is illustrated in Fig 1

## Results

Muscle glycogen content and total GS (I+D form) and HK activity were higher in the T legs (Table II) Glycogen content at rest averaged 81 mmol × kg $^{-1}$ in the U legs and was 32 % higher (average 119 mmol × kg$^{-1}$ ) in the T legs At this time both total GS and HK activity in the U legs averaged 2 8 $\mu$mol × g$^{-1}$ × min$^{-1}$ With training there was an 35 % and 18 % increase respectively in the activity of these enzymes (Table II)

The exercise performed on Day 1 resulted in a decrease in muscle glycogen content of the same magnitude in T and U legs which averaged 71 and 64 mmol

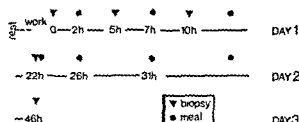



Fig 1 Time meal and sampling schedule The second sample was taken 5 min after exercise

kg⁻¹ respectively (Table II) The U legs had the lowest glycogen content both at the start of and after exercise Both T and U legs showed an increase in the percentage of GS present in the active I form (GSI) increasing from an average of 16 % at rest to 48 % after work (Table II and Fig 2) For the four one leg trained subjects GSI appeared to be higher in the U legs than in the T legs at rest This difference was not consistent after exercise Total GS activity and HK activity did not change with exercise nor during the 46 h recovery phase Only values from rest 0 and 46 h are shown in Table II Total GS and HK activity was very stable even at the other sampling times the total range for ecah subject was less than 3 8 %

TABLE II Individual and mean values ± S E for glycogen content total glycogen synthetase synthetase I form and hexokinase activity in trained (T) and untrained (U) leg muscle at rest 5 min and 46 h after exercise

| Subject | | Glycogen (glucose units) mmol kg⁻¹ | | | Glycogen synthetase: Total µmol × g⁻¹ min⁻¹ | | | Glycogen synthetase: I form % of total | | | Hexokinase µmol g⁻¹ min⁻¹ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Rest | After exercise 5 min (0 h) | After exercise 46 h | Rest | After exercise 5 min (0 h) | After exercise 46 h | Rest | After exercise 5 min (0 h) | After exercise 46 h | Rest | After exercise 5 min (0 h) | After exercise 46 h |
| KG | T | 112 | 45 | 130 | 3 2 | 3 0 | 3 2 | 11 7 | 53 3 | 10 0 | 3 3 | 3 1 | 3 5 |
| | U | 65 | 14 | 9? | 2 4 | 2 3 | 2 4 | 21 8 | 52 9 | 17 2 | 2 7 | 2 7 | 2 5 |
| SR | T | 153 | 66 | 128 | 3 6 | 3 8 | 3 5 | 4 7 | 40 6 | 10 3 | 3 0 | ?9 | 3 1 |
| | U | 115 | 26 | 88 | 2 5 | 2 5 | 2 5 | 7 1 | 56 5 | 13 7 | 2 7 | ?5 | 26 |
| JG | T | 117 | 40 | 120 | 5 5 | 5 1 | 5 3 | 15 8 | 55 2 | 13 4 | 4 2 | 4 2 | 4 0 |
| | U | 70 | [illegible] | 85 | 2 8 | 2 8 | 2 6 | 23 9 | 39 9 | 27 1 | 3 5 | 3 6 | 3 7 |
| SH | T | 127 | 47 | 135 | 4 2 | 4 0 | 4 0 | 12 8 | 60 0 | 20 1 | 3 2 | 3 1 | 3 0 |
| | U | 85 | 25 | 81 | 3 0 | 3 1 | 3 0 | 21 9 | 37 1 | 17 8 | 2 6 | 2 4 | 2 5 |
| BS | T | 85 | 17 | 3 | 4 8 | 4 9 | 4 5 | 19 3 | 35 5 | 10 0 | 3 3 | 3 5 | 3? |
| NM | U | 70 | 11 | 60 | 3 1 | 3 0 | 3 2 | 16 5 | 46 5 | 10 1 | 2 6 | 2 6 | 2 5 |
| Mean | T | 119 ± 11 | 43 8 | 117 11 | 4 3 ± 0 4 | 4 2 ± 0 4 | 4 1 ± 0 4 | 12 9 ± 2 4 | 48 9 ± 4 6 | 12 8 ± 1 9 | 3 4 ± 0 7 | 3 4 ± 0 2 | 3 4 ± 0 2 |
| Mean | U | 81 ± 9 | 16 ± 4 | 81 6 | 2 8 ± 0 1 | 2 7 ± 0 2 | 2 7 ± 0 2 | 18 2 ± 3 0 | 46 6 ± 3 7 | 17 2 ± 2 8 | 2 8 ± 0 ? | 2 8 ± 0 ? | 2 8 ± 0 ? |

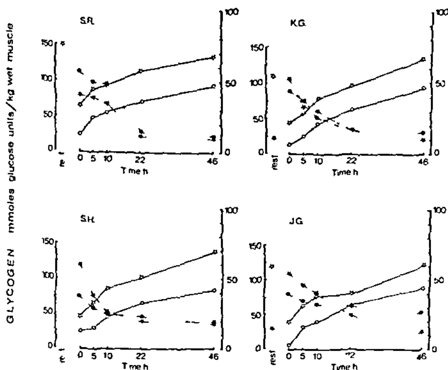



Fig 2 Variations in total muscle glycogen (☆ T and O U) and GSI activity (★ T and ● U) in trained (T) and untrained (U) leg muscle in the experiment designed in Fig 1

The biochemical results described were well supported by the simultaneous histochemical analyses Darker staining intensity for total GS (indicating enhanced activity) was found in the T leg with no difference between the ST and FT fibres This intensity was unaffected by exercise and remained unchanged throughout the experiment GSI activity varied drastically however At rest this activity was low as indicated by a pale stain for both legs and for the two fibre types After exercise when glycogen content was low staining intensity increased strikingly and was most pronounced in ST fibres with no difference between legs There was a gradual decrease in intensity during the recovery period and the difference between ST and FT fibres was clearly visible in the first 10 h After 46 h no difference could be detected as compared to the pre-exercise sample The staining patterns described for synthetase I form and glycogen are illustrated in Fig 3 with micrographs from one subject

Glycogen replenishment was a relatively slow process despite of the CHO enriched diet (Fig 2 and 4) In both legs glycogen accumulation was fastest during the first 10 h averaging 2 4 $\text{mmol} \times \text{kg}^{-1} \times \text{h}^{-1}$ During this period GSI values were higher than in the following 36 h (Fig 2) In agreement with the histochemical results GSI almost decreased to pre exercise values after 22 and 46 h but an ac

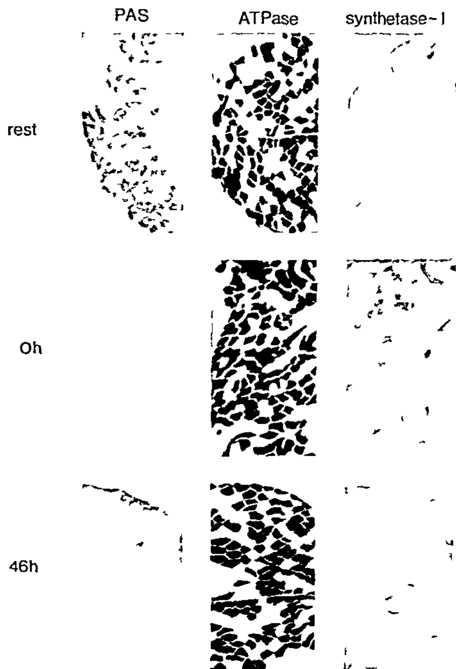



Fig 3 Histochemical staining patterns for PAS myofibrillar ATPase and GSI form from the L leg of subject JG A dark stain indicates enhanced enzyme activity Fibres stained dark for myofibrillar ATPase are referred to as FT fibres lightly stained fibres as ST fibres

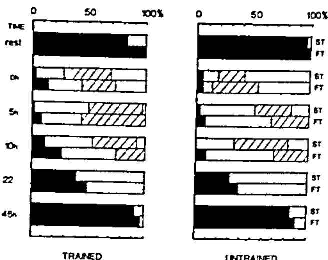



Fig 4 Subjective evaluation of PAS staining in ST and FT fibres for all subjects The 4 staining categories used were dark, moderate light and negative For each period the evaluation is based on at least 400 fibres

cumulation of glycogen still occurred at the average rate of 1 3 mmol × kg⁻¹ × h⁻¹ during the final 36 h The replenishment curves were strikingly parallel throughout the experiment with no indication of an increased rate of synthesis after training as shown in Fig 2 This figure includes the four one leg trained subjects Subjects BS and NM (two-leg trained and untrained) did not deviate from the pattern described above as evident from Table II

At rest the glycogen distribution pattern (PAS staining intensity) only displayed minor and inconsistent differences between the two fibre types Although not notable in Fig 4 the dark staining was more intense in trained muscle than in untrained muscle indicating a higher glycogen level After exercise on Day 1 almost all fibres were glycogen-depleted with some preponderance for ST fibres At this time 58 % of the ST and 46 % of the FT fibres in the U leg were rated negatively stained for glycogen as compared to 32 % ST and 27 % FT in the T leg This correlates well with the lower glycogen levels demonstrated biochemically in the U legs (Table II) During the restoring process no histochemical difference could be detected between the two legs in the rate of glycogen accumulation as was also the case for biochemical assay (Fig 2 and 4) The refilling of glycogen stores was almost complete after 46 h (Fig 3 and 4) As judged on the basis of PAS staining intensity there was no clear-cut difference in glycogen replenishment capacity between the two fibre types

## Discussion

The higher glycogen levels found in trained muscles have been focused on by many investigators (Gollnick *et al* 1972 a 1973 a) In previous studies it has been difficult to distinguish the effects of training from local effects in the muscle itself Vari

ations in diet, hormonal secretion and systemic circulatory adjustments could be possible explanations for the increase in muscle glycogen with training. In the present study simultaneous measurements were made on the legs of subjects who had only trained one of their legs, the other leg serving as a control. Higher glycogen levels were found in T legs than in U legs of the same subjects. Therefore training can now be said to induce a local increase in muscle glycogen, independently of possible dietary adjustments.

The mechanism behind the increase in muscle glycogen resulting from training was found to involve increased GS enzyme activity demonstrated both biochemically and histochemically. Similar biochemical results are available (Taylor, Thayer and Rao 1972). This increased glycogen synthesis capacity was not revealed during the restoring period following exercise-induced glycogen depletion. Replenishment curves for T and U legs were strikingly parallel. Immediately after glycogen depletion the U leg was almost glycogen depleted, whereas the T leg still contained about one third of its resting value (Fig. 2). It is possible that a higher rate of glycogen accumulation in T leg compared to U leg would have been found if both legs had contained equally low levels of glycogen after the depleting exercise. However, it was found very difficult to get the subjects to continue to exercise their T legs after exhaustion in order to further decrease the glycogen content.

As previously reported the restoring process is relatively slow and glycogen stores are not replenished until 46 h (Piehl 1973). Similar results were obtained in this study. In a study by Bergström and Hultman (1966) the restorage was completed after one day. The discrepancy can scarcely be explained by a difference in the diet as the same amount of kcal derived from CHO was used in both studies, namely 35 kcal · kg$^{-1}$ b.wt. · day$^{-1}$. The mode of GS enzyme activation during the enhanced glycogen synthesis following exersise was biochemically and histochemically investigated. Five minutes after end of exercise a pronounced increase was found in the levels of the active I form of the enzyme. Since there was no change in total GS activity, this increase must have been produced by a conversation of less active D form into more active I form. Such an effect has been previously described after exercise and muscular contractions (Hultman, Bergström and Roch-Norlund 1971, Adolfsson 1973). The decrease in I form during glycogen accumulation is most probably explained by the well known inverse relationship between GSI and glycogen (Danforth 1965, Adolfsson, Isaksson and Hjalmarson 1972, Bergström, Hultman and Roch-Norlund 1972). During the last 24 h of the 46 h recovery period, GSI almost declined to pre-exercise levels. Despite of the low GSI glycogen continued to accumulate during these last 24 h. Enhanced glycogen synthesis in the presence of low GSI level has been described previously (Adolfsson 1973a). G-6-P is both one of the substrates in glycogen synthesis and an activator of the GS enzyme. Therefore HK [illegible] synthesis by increasing the intracellular G-6-P pool (Hultman, Bergström and Roch-Norlund 1971). An increase in HK was found in T leg and [illegible] previous studies on guinea pigs where it was more pronounced [illegible] white (FT) muscles (Peter, Jeffres

and Lamb 1964 Lamb et al 1969) For humans however the reports concerning GS activity are more conflicting Engel (1962) found greater staining intensity for total GS enzyme in type II (FT) fibres and St George Stubbs and Blanchaer (1965) reported a greater intensity in red (ST) fibres No previous report of histochemical GSI analyses in human skeletal muscle appear to be available In the present study it was very interesting to note a greater staining intensity for GSI after exercise which was more pronounced in ST fibres The difference in GSI between fibre types may be explained by the above mentioned inverse relation between GSI and glycogen To fully clarify if there exist differences in the glycogen synthesis capacity between muscle fibres complementary studies on isolated muscle cells may be required

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# Effect of Ischemia on Monoamine Metabolism in the Brain

By

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Recent animal experiments demonstrate that considerable restitution of cerebral function and metabolism can occur even after prolonged interruption of cerebral circulation (for literature review see Ljunggren *et al* 1972 1974 b) However since signs of restitution of energy metabolism appear before the EEG returns towards normal patterns and long before neurological function is restituted it appears that tissue alterations other than energy failure underlie the functional deficits The ratelimiting steps in the synthesis of indole and catechol neurotransmitters are oxygen dependent and recent work has shown a deranged monoamine metabolism (Davis and Carlsson 1973) and a disruption of behaviour maintained by catechol amines (Brown *et al* 1973) as a result of hypoxia The present communication gives a preliminary account of the metabolism of catechol and indole amines in the brain during total cerebral ischemia and following restitution of the cerebral circulation

Male Wistar rats weighing 300—400 g were maintained immobilized and artificially ventilated on 70 % $N_2O$ and 30 % $O_2$ to give arterial CO tensions of 35—40 mm Hg and $O_2$ tensions exceeding 100 mm Hg Cerebral ischemia was induced by infusing an artificial CSF prewarmed to body temperature via a double barreled needle inserted into the cisterna magna until the CSF pressure exceeded the systolic arterial blood pressure (see Ljunggren *et al* 1972 1974 a) In one group interruption of cerebral circulation was maintained for 7 1/2 min and in another the ischemic phase was followed by a 30 min restitution period A control group was obtained by perfusing artificial CSF for 7 1/2 min through the double barreled needle without causing an increase in CSF pressure and the animals were left for another 30 min In all groups the tissue was frozen *in situ* for further biochemical analyses at the end of the experimental periods Blood pressure and CSF pressure were continuously monitored and arterial blood was repeatedly sampled for analyses of pH $P_{CO}$ and $P_O$ The body temperature was maintained close to 37 C and the brain temperature was prevented from falling

Brains were homogenized in 10 ml of 0 4 ml of 0 4 N perchloric acid containing 5 mg $Na_2S_2O$ and 20 mg EDTA After centrifugation and neutralization of the extracts they were eluted on a Dowex 50 X-4 cation exchange column and spectrophotofluorimetric analyse were made on the eluates for noradrenaline 5 hydroxytryptamine (5-HT) 5 hydroxyindol acetic acid (5 HIAA) and tryptophan (for references to individual methods see Davis and Carlsson 1973)

As described elsewhere (Ljunggren *et al* 1972 1974 a) 7 1/2 min of complete cerebral ischemia leads to disappearance of glucose phosphocreatine and ATP and to an increase in the lactate content of the tissue to about 14 µmol/g However

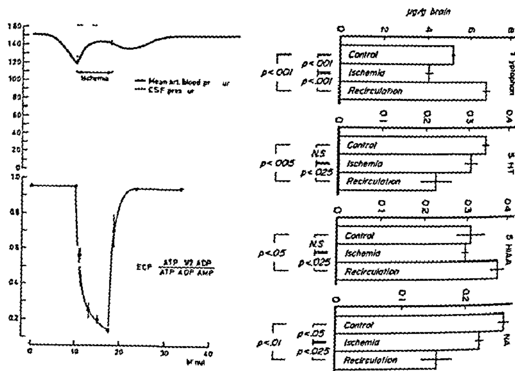


Fig 1 Upper panel Mean arterial blood pressure and intracranial CSF pressure in mm Hg during 7 1/2 min of ischemia and following recirculation Lower panel Adenylate energy charge (ECP) of brain tissue calculated as shown in the figure The values for ECP were taken from Ljunggren *et al* (1974 a and b) Values for ECP are given ± S E

Fig 2 Mean whole brain levels of tryptophan, 5 hydroxtryptamine (5 HT) 5 hydroxyindole acetic acid (5 HIAA) and noradrenaline in rats exposed to 7 5 min of ischemia and after a 30 min period of cerebral recirculation following 7 1/2 min of ischemia. Values are given as μg/g brain ± S E (N = 4 animals per treatment)

recirculation of the tissue following the ischemia rapidly restores phosphocreatine and allows rephosphorylation of the adenine nucleotide pool The rapid normaliza tion of the adenylate energy charge (Fig 1) demonstrates that oxidative phospho-rylation is resumed and that no significant part of the tissue remains unperfused in the restitution period

Indole levels were not appreciably altered during the 7 1/2 min of ischemia al though there was a trend towards a decrease in serotonin (5 HT) levels (Fig 2) During recirculation there was a significant drop in 5 HT level and a significant increase in its deaminated metabolite 5 HIAA Brain tryptophan showed a significant decrease during ischemia and a significant increase during recirculation Brain noradrenaline concentration was significantly reduced during the time of ischemia and this decrease continued during the recirculation period

The present results show that there is a persisting disturbance in monoamine meta bolism 30 min after an ischemic insult of 7 1/2 min duration in spite of the fact

that the energy state of the brain has been almost completely restored The therefore indicate that transmission failure rather than energy failure may be cause of the functional deficits that persist after the ischemia

The ischemic model used in this study leads to almost instantaneous interruption of the cerebral circulation and total oxygen lack is therefore obtained within a few seconds Energy stores in the form of ATP are depleted within a few minutes and lactic acid production and CO retention contribute to give a marked cellular acidosis The combination of anoxia energy failure and acidosis may explain many of the present findings In the present context it should only be recalled that oxygen lack does not only inhibit tyrosine and tryptophan hydroxylases (see Davis and Carlsson 1973) but also monoamino oxidase (MAO) hence marked changes in levels of catechol and indole neurotransmitters cannot be expected to occur during the ischemia However since the noradrenaline storage mechanism requires high energy phosphates (Euler and Lishajko 1963) and is disrupted by marked decreases in pH (Euler and Lishajko 1961) it is conceivable that the ischemia releases monoamines from the granules and that the reoxygenation of the tissue after the ischemia accelerates loss of *e g* noradrenaline by means of an activation of MAO Ischemia may also cause leakage of transmitter through the cell membrane since the maintenance of membrane potentials and transmitter reuptake are ATP-dependent Monoamine synthesis storage and release processes during and after ischemia merit further study

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# Influences on Gastrointestinal and Bladder Motility by the Fastigial Nucleus

By

BJORN LISANDER and JAN MARTNER

Although the overwhelming amount of physiological studies on the cerebellum have been devoted to its somatomotor functions there have been several reports on autonomic influences (*cf* Dow and Moruzzi 1958) emanating from this structure Thus marked increases in blood pressure can be elicited by stimulation of the rostral pole of the fastigial nucleus (Miura and Reis 1969) and recently several studies have dealt with this cardiovascular response (Achari and Downman 1970 Lisander and Martner 1971 1973) which is characterized by widespread sympathetic activation of heart and vessels as well as by an inhibition of prevailing cardiac vagal tone Quite recently there have been reports on cerebellar effects on gastric and duodenal motility as well (Beller and Talan 1971 Manchanda *et al* 1972)

It was considered of interest to study to what extent and in which ways the activity of autonomically innervated organs other than the cardiovascular system is influenced by fastigial stimulation and if differentiated effects could be traced The experiments were performed on chloralosed cats with recording of blood pressure heart rate bladder pressure and gastric and intestinal motility Gastric motility was measured by recording volume changes in an intragastric balloon at low constant pressure Intestinal motility was followed by either recording pressure changes in an intraluminal balloon or the volume changes of isolated intestinal loops In all experiments where intestinal motility was recorded the adrenal catecholamine secretion was eliminated by denervation and/or ligation of these organs

Fastigial stimulation was found to influence all parameters recorded Gastric tone could either increase or decrease and sometimes a biphasic response was obtained with an initial relaxation followed by a contraction The colonic response upon stimulation was always excitatory and the effective fastigial structures corresponded well with those eliciting blood pressure rises although occasionally colonic contractions could be elicited without any concomitant pressor response In the small intestine activity could either be increased or decreased however with some regional differences Thus the ileum responded predominantly with an increased tone while the jejunum showed a mixed response pattern with inhibition in some experiments and excitation in others As can be seen from the figure the same topical stimulation of the fastigius nucleus can elicit contraction of the colon and ileum and inhibition

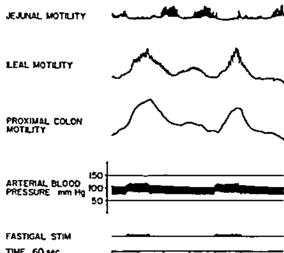



Fig 1 Cat 2 9 kg chloralose Adrenals ligated and hydrocortison 5 mg/kg b w given Fastigial stimulation (50 Hz, 1 ms 0 2 mA) produces a moderate pressor response Note the increased motility and tone in colon and ileum while there is a prompt inhibition of spontaneous jejunal motility

of jejunal motility and this response pattern was the most commonly encountered one Histological examination revealed the responsive structures to be located in the rostral fastigial pole or the just rostrally situated cerebellar white matter

The peripheral mechanisms mediating these responses were subject to analysis If the inhibitory sympathetic influence on the colon was initially abolished by either guanethidine (Ismelin® Ciba) or by lumbar colonic nerve section the colon exhibited a spontaneous marked peristaltic activity which was in no way influenced by fastigial stimulation If on the other hand the parasympathetic pathways were initially interrupted by section of the vagi and the pelvic nerves leaving the sympathetic supply intact the excitatory effect by fastigial stimulation was still present Atropine reduced but only occasionally abolished the colonic contractions induced by fastigial stimulation Likewise the excitatory response of the small intestine induced by fastigial stimulation was completely abolished by guanethidine but not by bilateral vagotomy although it was sometimes reduced after the latter procedure Thus the fastigial excitatory influence on intestinal motility appears to be mediated mainly by changes in sympathetic tone and since the sympathetic influence is here generally of inhibitory type the results suggest a suppression of a prevailing tonic activity of the sympathetic intestinal supply However these responses must be subject to further analysis

Filling the bladder with saline induced regularly an increase in its motility and this response was completely inhibited during fastigial stimulation The effective fastigial parts were quite large and not restricted to those eliciting pressor responses only Furthermore since this inhibition persisted after guanethidine it suggests that the fastigial pathways suppress the efferent parasympathetic discharge presumably by an inhibitory action at the spinal level In a few experiments an increased bladder motility was observed during fastigial stimulation but then the responsive part of the nucleus was considerably smaller

The present findings indicate that the fastigial nucleus may exert effects on the motility of the intestinal tract by modulating primarily the activity in the regional sympathetic nerves This occurs in the direction of suppression which is opposite to the fastigial effects on the adrenergic cardiovascular supply which is mainly excitatory (Lisander and Martner 1971) Presumably the modulation of the sympathetic gastrointestinal supply described here is related to that producing suppression of the intestino intestinal reflex *via* supraspinal structures such as the neuron pool in the medullary depressor area (Johansson Jonsson and Ljung 1965) Whatever the mechanisms the present study has indicated that the fastigial nucleus and hence the cerebellum affects not only the cardiovascular system but can also considerably modulate the activity of other autonomically innervated organs like the gastrointestinal tract and the bladder These effects seem to be *predominantly though not exclusively mediated via differentiated modulations of* sympathetic discharge which warrants further detailed analysis

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# Spindle Responses in Pig Eye Muscles

By

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Muscle spindles exist in the eye muscles of different species including pig sheep and man (Cooper Daniel and Whitteridge 1955) Recent studies have shown that spindles in sheep eye muscles are morphologically rather similar to those in for example cat limb muscles (Harker 1973) Also eye muscle spindles seem to have nuclear bag and chain fibers primary and secondary endings and fusimotor innervation of two kinds However a physiological demonstration of primary and secondary afferents or of dynamic and static fusimotor effects (Matthews 1964) had yet to be delivered and this is the purpose of the present study

In mini pigs (14—25 kg) recordings were made with tungsten microelectrodes from single ganglion cells of eye muscle spindle afferents in the Gasserian ganglion (Manni Bartolami and Desole 1968) The trochlear and the oculomotor nerves were transected The individual oculorotatory muscles were connected to a muscle puller described by Collins (1971)

Spindle endings were identified from the reduction in firing during muscle twitch

As a method of differentiating between primary and secondary endings sinusoidal vibrations of high frequencies were applied primary endings can follow higher frequencies of vibration than secondary endings (Bianconi and van der Meulen 1963 Brown Engberg and Matthews 1967)

The muscles were also stretched and released along a triangular curve the frequency of which varied between zero and 16 Hz The peak to peak amplitude was 4 mm Static and dynamic responses were determined from spindle afferent impulse frequency—muscle length diagrams (f ΔL diagrams) according to the methods described by Lennerstrand (1968 *a*)

The ending of Fig 1 could follow vibration at 225 Hz and the ending of Fig 2 followed 475 Hz The f ΔL diagrams of Fig 1 A and Fig 2 A have the shapes of those obtained in cat spindle secondary and primary endings respectively (Lennerstrand 1968 *a*) Further the ending of Fig 2 ceased firing during release of stretch at 2 Hz which is highly characteristic of primary endings The dynamic response

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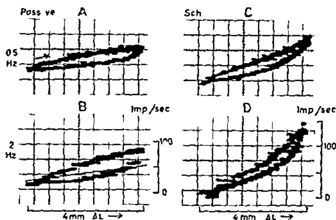



Fig 1 f ΔL diagram of the responses of a secondary ending subjected to triangular length changes of 4 mm/s (A and C) and 16 mm/s (B and D) Afferent instantaneous impulse frequency on the ordinate and muscle length change on the abscissa Solid line in B is steady state curve Arrow indicates phase lead in spindle response over muscle length change A and B before C and D two min after the i v injection of 250 μg/kg b w Sch Note reduction in dynamic response i e decrease in f ΔL loop width and increase in slope of the loops as an effect of the drug

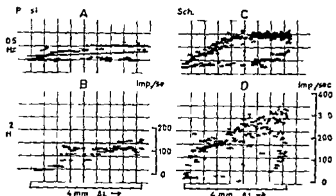



Fig 2 f ΔL diagrams of a primary ending during the same experimental conditions as in Fig 1 At 16 mm/s before injection of Sch (B) the ending fired very few impulses during release of stretch which is typical of primary endings The effect of Sch was in this case to increase the dynamic response (C and D) Figures retouched

was also larger in the ending of Fig 2 than in the ending of Fig 1 which is further support of the notion that the former was a primary and the latter a secondary ending

Succinylcholine (Sch) is known to accelerate spindle firing by contracting the intrafusal fibers (Smith 1966) In primary endings the most long lasting effects resemble those of dynamic fusimotor single fiber activation (Lennerstrand 1968 b) Injection of Sch increased the dynamic response also in primary endings of pig eye muscles (Fig 2 B) indicating dynamic fusimotor control In secondary endings on the other hand the dynamic response was reduced but the position sensitivity (i e the slope of the f ΔL diagram) was increased (Fig 1 B) These are typical static fusimotor effects (Lennerstrand and Thoden 1968) It is known that dynamic fusimotor innervation occurs only in primary endings of cat spindles while static fusimotor effects are seen in both primary and secondary endings (Matthews 1964)

Thus, these functional studies have demonstrated primary and secondary afferent responses and dynamic and static fusimotor innervation in the eye muscle spindles of the pig Whether the spindle machinery is used in the same way in the oculomotor control as in the spinal motor control (Matthews 1972) has yet to be determined

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# Effect of Human Menopausal Gonadotrophin on Amino Acid Transport in the Prepubertal Rat Ovary

By

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Previous experiments have shown that intravenous (i v ) injection of various ovine FSH preparations to prepubertal rats shortly before removal of the ovaries stimulates *in vitro* ovarian uptake of the non utilizable amino acids α amino isobutyric acid (AIB) and 1 amino-cyclopentane carboxylic acid Injection of these FSH preparations also stimulates the uptake and incorporation into ovarian protein of several normal amino acids *e g* glycine valine leucine and proline and this stimulation has always been correlated to an increased rate of AIB uptake (Ahren *et al* 1969)

The present study was undertaken in order to explore whether also FSH preparations from other species are effective in this experimental system Such a study seems now even more important since it has recently been established that LH preparations also can stimulate amino acid uptake and incorporation in the prepubertal rat ovary (Nilsson and Selstam to be published) FSH preparations extracted from menopausal urine have been used in the present study because precipitation of these preparations with anti HCG serum can decrease the LH contamination to undetectable levels (Donini *et al* 1966) In the present experiments ovarian lactate production was studied in addition to ovarian AIB uptake and a comparison was made with an ovine FSH preparation

Both 24 day old normal female rats and 42 day old hypophysectomized rats were used The hypophysectomized rats were operated on when 28 days old After the hormone injections ovaries were taken out dissected free from extraneous tissues and incubated for 2 h in Krebs bicarbonate buffer containing 5 5 mM glucose 0 1 mM AIB 1 ¹⁴C (0 1 μCi/ml) and 0 07 mM sucrose ³H (2 5 μCi/ml) Lactic acid accumulation in the medium was measured according to Lundholm *et al* 1963) Distribution ratios of AIB were determined as described earlier (*e g* Ahren and Kullander 1965) In calculating the distribution ratio sucrose space was considered to represent the extracellular space

Ovine FSH (NIH FSH S ) as supplied by the Endocrinology Study Section of the National Institutes of Health NIH USA FSH and LH activities of this FSH preparation were determined by NIH with the assay of Steelman and Pohley (1953) and with the ovarian ascorbic acid depletion (OAAD) test Parlow 1961) respectively The activities of this preparation after transformation into international units (IU) according to Reichert and Parlow (1964) and Donini *et al* (1966 was 29 4 IU FSH and less than 15 4 IU LH per mg Two HMG preparations ( 1 43 17 4 and 1 71) were prepared from postmenopausal urine mainly through the kaolin method of Albert *et al* (1961) A partial separation of FSH and LH was achieved by chromatography on diethylaminoethyl cellulose (DEAE C) twice for HMG 1 71 followed by chromatography on carboxymethyl cellulose HMC L 93 was also run twice on DEAE C with an anti HCG precipitation (Donini *et al* 1966) in between

TABLE I Amino acid uptake and lactate production in ovaries from normal and hypophysectomized prepubertal rats injected with various FSH preparations

| Hormone | | Normal rats | | | | Hypophysectomized rats | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | AIB-$^{14}$C distribution ratio | | Lactate production | | AIB $^{14}$C distribution ratio | | Lactate production | |
| Saline | | 13.1 ± 0.4 | (7) | 124 ± 9 | (7) | 16.2 ± 0.8 | (6) | 239 ± 17 | (6) |
| HMG I 71 | 50 | 15.5 ± 0.8** | (7) | 248 ± 21** | (7) | – | | – | |
| | 150 | 16.1 ± 0.5* | (6) | 328 ± 15 * | (6) | 23.2 ± 0.3 | (6) | 775 ± 70 | (6) |
| HMG L 93 | 100 | 16.1 ± 0.1** | (6) | 208 ± 10* | (6) | – | | – | |
| | 350 | 17.0 ± 0.7 * | (6) | 248 ± 22** | (6) | 19.7 ± 0.6 | (6) | 526 ± 32 | (5) |
| FSH S9 | 50 | 15.0 ± 0.5* | (6) | 189 ± 10** | (6) | – | | – | |
| | 500 | 16.3 ± 0.6 * | (5) | 268 ± 8 | (5) | 23.3 ± 1.3 * | (6) | 582 ± 29 * | (6) |

Hormones (μg/100 g b.wt.) were injected i.v. in 0.5 ml of 0.9 % NaCl/100 g b.wt. under light ether anesthesia 2 h before sacrifice. Ovaries were incubated for 2 h in Krebs bicarbonate buffer containing 5.5 mM glucose and 0.1 mM AIB-$^{14}$C. The AIB distribution ratio was calculated as CPM per ml intracellular water/CPM per ml medium and lactate production as μg/100 mg tissue wet weight. Means ± S.E. with numbers of observations within brackets. Significances as compared to controls with analysis of variance. * = $p < 0.05$, ** = $p < 0.01$.

HMG I 71 contained 179 IU of FSH and 6 IU of LH and L 93 held 75 IU FSH and undetectable LH activity with Steelman Pohley and OAAD assays. Statistical significances as compared to controls were calculated with analysis of variance followed by Student-Newman-Keuls multiple range test (Woolf 1968). A p value less than 0.05 was considered significant.

The results are summarized in Table I. The two HMG preparations stimulated AIB uptake in a manner similar to the ovine FSH preparation. One of these preparations (L 93) had been precipitated with an anti HCG serum and contained no detectable amounts of LH activity. The results demonstrate therefore not only that human FSH can stimulate ovarian amino acid uptake but also that FSH has this effect without a significant LH contamination. Table I shows that this is true also for the effect of FSH on ovarian lactate production which confirms earlier observations (for ref. see Ahrén *et al.* 1969). The slightly smaller effects of the very pure HMG preparation (L 93) in the hypophysectomized rats might indicate a synergism between FSH and LH on both amino acid transport and glycolysis. Whether FSH and LH exert their effects in the same cell type(s) or in different cell types of the ovary is one of the questions currently under investigation in this laboratory.

For generous gifts of hormone preparations our thanks are due to docent Torsten Perklev, Leo Ltd, and to NIH. Valuable suggestions were offered by professor Kurt Ahrén. Expert technical assistance was given by Miss Stina Öberg and Mrs Ann Cathrine Schiött. This work was supported by grants from US Public Health (2 RO1 HD02195) from the Swedish Medical Research Council (B74 03X 27) and from the Faculty of Medicine, University of Göteborg.

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